

REMARKS

I. Status of the Claims

Claims 1-27 are currently pending in the present application. Claims 12-21 and 23-25 are currently withdrawn. Claim 2 has been amended to correct a typographical error where two words ran together. Claims 2, 3, and 22 have been amended for clarity. Instead of reciting "the selectable marker and/or the unpaired splice donor", the claim has been amended to "the selectable marker or the unpaired splice donor or both", according to the Examiner's suggestion. Claim 11 has been amended to recite that the isolated cell is cloned in order to narrow the scope from dependent claim 4. Claims 26 and 27 have been amended to refer back to the cell rather than the vector. Claim 26 has been amended to correct a grammatical error. Accordingly, no new matter has been added with the amendment of these claims.

II. Examiner Interview

A. Issues in the Interview

Applicants thank Examiner Chen for the helpful telephonic interview held with Applicants' attorney, Anne Brown, on July 21, 2008. The substance of the interview was directed to the rejection of the claims as not enabled. There were two bases for the rejection. The first was the Examiner's position that a splice donor was necessary for producing a fusion transcript of endogenous gene and exogenous vector sequence. See the statement on page 6 of the Office Action. In this regard, Applicants presented three attachments (A., B., and C.). Attachment C was an abstract of Niwa et al. (J. Biochem., 113:343-349 (1993)) in which a fusion transcript was produced from a vector that contained a reporter gene and a promoter but no splice donor. Attachment B was a schematic diagram of Niwa et al. Applicants' attorney pointed out that transcription would begin from the vector promoter, proceed through the reporter gene, proceed into the genomic DNA, and end at a polyadenylation signal in the genomic DNA.

Applicants' attorney also presented various possible configurations for the Applicants' claimed vector in Attachment A. For example, in Figure A, transcription can proceed from either promoter on the vector and will continue through the splice donor site into a single exon or into exon 1. Accordingly, transcription would proceed until it reached a genomic polyadenylation signal. Then it would terminate. From Figure A, it is obvious that transcription need not involve splicing. Figure B has a different configuration of the vector and diagrams a single exon gene or exon 1. Again, two transcripts would be produced from either promoter, but splicing would not occur.

Figure C shows a double exon endogenous gene where splicing can occur in two ways: (1) by splicing from the splice donor in exon 1 to the splice acceptor in exon 2, and (2) by splicing from the splice donor on the vector onto exon 2. In this case, exon 1 would be spliced out. Figure D shows a different configuration of Applicants' claimed vector wherein a larger area of the transcript would be spliced out when splicing occurs from the splice donor on the vector to the splice acceptor on exon 2.

But, in all cases, transcription will precede from a promoter on the vector, through the vector, into the genomic DNA, and will terminate when the polymerase encounters a genomic polyadenylation signal. It is evident from these diagrams that splicing is not necessary to produce a fusion transcript. All that is necessary is a promoter on the vector without an operable polyadenylation signal on the vector. This is exemplified by Niwa et al., which is provided in its entirety as Attachment C.

Applicants' attorney also discussed the statement on page 6 of the Office Action that only a retrovirus can integrate into the genome by non-homologous recombination. Applicants' attorney explained that recombinant gene expression is based on introducing cloned genes into eukaryotic cells. A retroviral vector is one way. But, in most cases, DNA is not introduced on a retroviral vector. It has been well-known, since the 1970s, that virtually any DNA sequence can stably (and randomly) integrate into a genome. This include random restriction fragments. See Attachment C as an example (Niwa et al.).

Here, an exogenous DNA sequence stably and randomly integrates into the eukaryotic genome. It is not introduced by retrovirus infection.

In the interview, the Examiner stated that it was his belief that introducing a gene into a cell was done by designing that gene to be on an extrachromosomal element, such as a plasmid. Applicants acknowledge that bacterial cloning was often done with plasmids that were replicated as extrachromosomal elements. This allowed easy purification of the lower density plasmid band. But, even in bacteria (which are not subject to retrovirus infection), exogenous DNA also integrates by non-homologous recombination into the bacterial genome. This occurs not only with linear plasmids introduced into the bacterial genome, but also with bacteriophage, for example.

In eukaryotic cells, DNA constructs not integrated into the chromosome are eventually lost ("transient transformant"). However, DNA integrated into the genome becomes a stable part of the genome ("stable transformant"). This procedure was used for many years during the development of recombinant DNA and gene expression in eukaryotic cells. Exogenous DNA that was designed to be introduced into a eukaryotic cell and remain outside of the genome as an extrachromosomal element had to be deliberately manipulated not to integrate. For example, the element would have to contain an autonomously replicating sequence. Otherwise, it would be simply be diluted out upon cell division and would essentially constitute a transient transformant.

Applicants attach Chapter 16 of a routinely-used cloning manual that is basic technical material for molecular biology laboratory manipulations (Attachment D). Chapter 16 discusses the introduction and expression of cloned genes in eukaryotic cells and presents several ways to introduce DNA that are unrelated to retroviruses. It is also evident from the reference list that many different kinds of vectors, unrelated to retroviruses, were used in these techniques. In fact, virus-mediated transduction was not even addressed in this chapter; the introduction indicates that viral methods of introducing DNA are in a separate chapter.

The Examiner is directed particularly to pages 16.2, 16.47, and 16.52 explaining transient and stable transformation with non-retroviral DNA. Page 16.4 and Figure 16.2 show transformation with plasmid vectors. Pages 16.14 and 16.21 show transformation with mere restriction fragments and sheared DNA (16.22). All these integrate randomly into the genome.

It should also be noted that stable transformants are easily detected by having a selectable marker. That explains the presence of a selectable or reporter gene in gene trap vectors such as Niwa et al.

B. The References (Attachments E1-E10)

In the interview, the Examiner requested that Applicants present evidence of random integration of non-retroviral DNA. The molecular cloning manual mentioned above cites several references showing DNA transformation by non-retroviral vectors and dating back to the 1970s and 1980s. In addition, Applicants have searched for other references, at the Examiner's request, showing non-homologous integration into the cellular genome by non-retroviral sequences, including DNA fragments.

Wigler et al. (Cell, 14:725-31 (1978)) was referenced in the manual. These studies show transforming a restriction fragment of the HSV genome containing the HSV thymidine kinase (TK) gene into cultured mammalian cells. As is shown, the TK gene was successfully integrated into the host cellular genome. It was present in high molecular weight DNA from the host cell. A secondary transformation was done using genomic DNA derived from the primary transformant. The TK activity could be transferred from the host cell genome to secondary transformants.

Pellicer et al. (Cell, 14:133-41 (1978)) also show introducing a restriction fragment containing the TK gene. This, by itself, could be used to successfully stably transform other cells, indicating that the gene was integrated into the genome of the host cell. *The reference indicates that integration is not site-specific and occurs at different loci in all of the transformants.*

Pellicer et al. (Science, 209:1414-22 (1980)) again shows that transformation of a different selectable function can be used to stably transform a host cell. *Again, the reference indicates that there were no unique chromosomal locations and that different lines containing the DNA show that it is integrated on different chromosomes.*

Gorman et al. (Science, 221:551-553 (1983)), which is cited in the molecular cloning manual, is cumulative with the work of the previous references. It shows that plasmid vectors carrying selectable markers can be introduced into eukaryotic cells where there is stable transformation. This indicates integration into the cellular genomic DNA.

Gorman et al. (Philos. Trans. R. Soc. Lond. B. Biol. Sci., 307:343-346 (1984)) further expands this study showing stable transformation of eukaryotic cells with DNA containing single gene functions.

Peterson and McBride (Proc. Nat'l. Acad. Sci. USA, 77:1583-87 (1980)) show DNA-mediated transfer of a selectable marker gene into a eukaryotic cell. The reference shows efficient transfer of cellular genes using purified high molecular weight DNA. The DNA sequences were not even on a plasmid. The reference shows stable transformation of the linked TK and GALK genes, indicating integration into the cellular genome. Peterson and McBride also references Wigler et al. as demonstrating that single copy eukaryotic genes can be transferred into mammalian cells using unfractionated total cellular DNA as evidenced by the successful transformation of the cellular TK gene.

Wold et al. (Proc. Nat'l. Acad. Sci. USA, 76:5684-5688 (1979)) shows the introduction of a restriction fragment containing the rabbit β -globin gene into eukaryotic cells and the stable integration of this gene into the recipient cell genome. Figure 2 shows the presence of the globin gene in high molecular weight DNA from the host cell.

Wigler et al. (Proc. Nat'l. Acad. Sci. USA, 77:3567-3570 (1980)) show the transformation into a recipient eukaryotic cell of a mutant DHFR gene where the gene was introduced on a restriction fragment from

total genomic DNA. The reference indicates that co-transforming any sequence with the DHFR gene should be reasonably successful.

Wigler et al. (Proc. Nat'l. Acad. Sci. USA, 76:1373-1376 (1979)) also show successful transformation using unfractionated high molecular weight genomic DNA from a donor cell. They show the stable transformation of the DNA sequence, indicating that it stably integrated into genomic DNA. On the final page of the document, the authors discuss, in general, transformation with restriction endonuclease-cleaved DNA.

Mulligan and Berg (Proc. Nat'l. Acad. Sci. USA, 78:2072-2076 (1981)) show the stable transformation of a bacterial gene into eukaryotic cells. The reference shows the DNA sequences in high molecular weight DNA extracted from the transformants (Figure 6).

All these references use the same technique: random non-viral transformation. All show that non-retroviral DNA can be integrated into the eukaryotic genome in a stable and random manner that does not necessitate using any retroviral infection protocol or any retroviral sequence.

III. Objections

The typographical error in claim 2 has been corrected. Claim 11 has been amended to recite that the cell is isolated and cloned. Accordingly, Applicants have overcome the objections and respectfully request that they be withdrawn.

IV. The Rejections

A. Rejection Under 35 U.S.C. § 112, Second Paragraph

On page 3 of the Office Action, claims 2-11, 22, 26, and 27 have been rejected under 35 U.S.C. § 112, second paragraph, on the grounds that they are indefinite. Applicants traverse the rejection.

Specifically, the Examiner objects that the phrase “the selectable marker and/or the unpaired splice donor” is indefinite. Without acquiescing in the propriety of the rejection, Applicants have adopted the suggestion of the Examiner, amending the claim to recite “the selectable marker or the unpaired splice donor or both”. Accordingly, Applicants submit that this rejection has been overcome. Reconsideration and withdrawal of the rejection is, therefore, respectfully requested.

B. Rejection Under 35 U.S.C. § 112, First Paragraph

On page 3 of the Office Action, claims 2-11, 22, 26, and 27 have been rejected under 35 U.S.C. § 112, first paragraph, on the grounds that they are not enabled. Applicants traverse the rejection.

First, the Examiner asserts (page 6 of the Office Action) that the application does not enable generating a fusion transcript without splicing of a splice donor (in the vector) into a splice acceptor. Applicants respectfully disagree. The Examiner is referred to the discussion above regarding the issue as discussed in the interview and the evidence presented.

As an example, as Attachment A to this Response, Applicants provide Figures A and B showing that a fusion transcript can be produced without splicing of the splice donor into a splice acceptor. This would be the case when the vector integrates into a gene upstream of the first exon (rather than in an intron). Transcription would begin with either of the promoters on the vector and would proceed through the splice donor sequence on the vector and into the first exon. If the gene is a single exon gene, splicing is irrelevant. If the gene is a dual exon gene, there will be endogenous splicing from the splice donor on the first exon to the splice donor on the second exon. There may also be splicing from the splice donor on the vector to the splice acceptor on the second exon. But, as is evident from these diagrams, depending on the nature of the gene into which the vector integrates, splicing may or may not be necessary from a splice donor on the vector.

On page 6 of the Office Action, the Examiner states that retroviral vectors can integrate into the genome with a retroviral DNA integration process that involves certain enzymes. The Examiner then makes the assertion "however, the state of the art shows that a vector other than retroviral vector cannot non-homologously integrate into the genome of a eukaryotic cell without the presence of a splice donor and a splice acceptor". Applicants respectfully disagree. The Examiner is referred to the discussion above regarding the issue as discussed in the interview and the evidence presented.

Attachment C (Niwa et al.) demonstrates that a non-retrovirus vector with a promoter but not a splice donor can (1) integrate by non-homologous recombination and (2) produce a fusion transcript with an endogenous gene. Applicants have also attached Attachments D and E1-E10 showing that non-homologous integration can be achieved with non-retroviral DNA.

For all these reasons, Applicants disagree with the statement on page 6 asserting that there is not sufficient guidance to generate a fusion transcript with one or more exons of an endogenous gene by non-homologous integration of a vector without the use of a splice donor.

Based on the above discussion, Applicants respectfully submit that the grounds for rejection have been addressed and the rejection overcome. Reconsideration and withdrawal of the rejection is, therefore, respectfully requested.

C. Rejection of the Claims on the Grounds of Double Patenting

On pages 7-16 of the Office Action, claims are rejected over various claims in the following U.S. patents: 7,033,782 (page 7); 6,740,503 (page 9); 6,623,958 (page 11); 6,602,686 (page 12); and U.S. Application No. 10/331,329 (page 14). Applicants respectfully traverse the rejection.

First, Applicants bring to the Examiner's attention that all of these patents and application are either divisionals or continuations of divisionals from the original parent application 09/276,820 (now U.S. Patent No. 6,897,066). Examiner Shukla issued a Restriction Requirement in that application. Applicants

point out that the '782 patent and '329 application are directed to vectors that do not necessarily contain two promoters. The '503 patent is directed to expressing a gene on genomic DNA and not by integrating the vector into a cellular genome. The '958 patent is directed to using positive and negative selectable markers. The '686 patent is generically directed to a vector with two promoters, two exons, and two splice donors, but not directed to the specific design of the vector in the present case. The Examiner is respectfully directed to the original Restriction Requirement to reconsider the rejection on the grounds of double patenting.

D. Rejection of the Claims Under 35 U.S.C. § 102

On page 16 of the Office Action, claim 27 is rejected as being anticipated by U.S. Patent No. 6,139,833, herein the '833 patent. Applicants respectfully traverse the rejection.

Claim 27 contains a typographical error in being directed to a vector. The claim has been amended to recite that the claim is directed to the cell of claim 2 or 3, where the vector contains the selectable marker selected from the recited group. In view of this amendment, reconsideration or withdrawal of the rejection is requested.

E. Rejection of the Claims Under 35 U.S.C. § 103

On page 17 of the Office Action, claim 26 is rejected as being obvious over U.S. Patent No. 6,139,833. Applicants respectfully traverse the rejection.

Claim 26 also contains a typographical error in being directed to a vector. The claim is now directed to the cell of claim 2 or 3, where the promoter on the vector is selected from the group consisting of the specific types of promoters. In view of this amendment, Applicants submit that the grounds for rejection have been overcome and respectfully request withdrawal of the rejection.

V. Conclusion

In view of the Applicants' amendments and discussion, Applicants believe that the pending claims are in condition for allowance. Early notification to that effect is respectfully requested. If it is believed that a further interview will expedite prosecution, the Examiner is invited to contact Applicants' attorney Anne Brown at 216-566-8921.

Applicants believe that fees for a three-month extension of time are due with this filing. Such fee is being simultaneously paid via electronic funds transfer with this submission. The Commissioner is hereby authorized to charge any additional fees required or to credit any overpayment to Deposit Account 20-0809. The applicant(s) hereby authorizes the Commissioner under 37 C.F.R. §1.136(a)(3) to treat any paper that is filed in this application which requires an extension of time as incorporating a request for such an extension.

Respectfully submitted,



Anne Brown
Reg. No. 36,463

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THOMPSON HINE LLP
10 West Second Street
2000 Courthouse Plaza, N.E.
Dayton, Ohio 45402
(216) 566-8921

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Attachment A

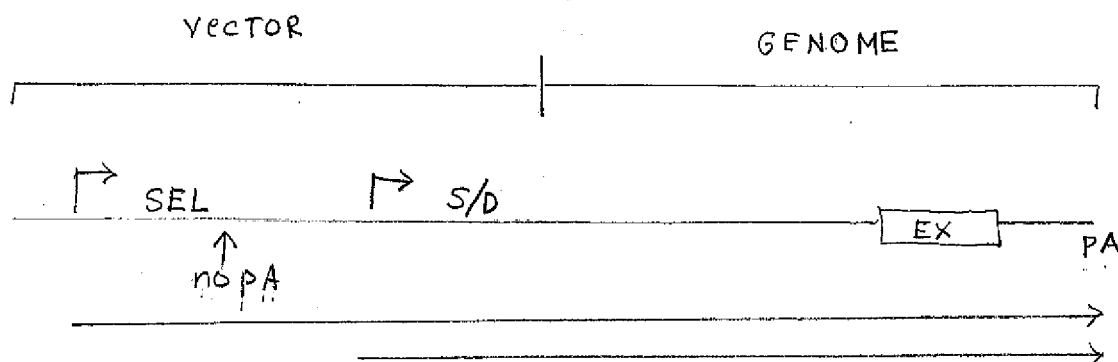


Fig. A.

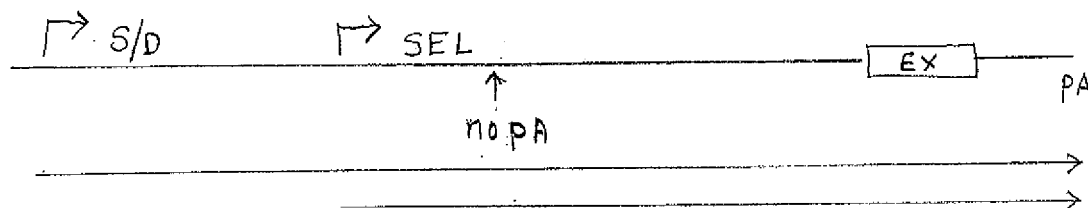


Fig. B.

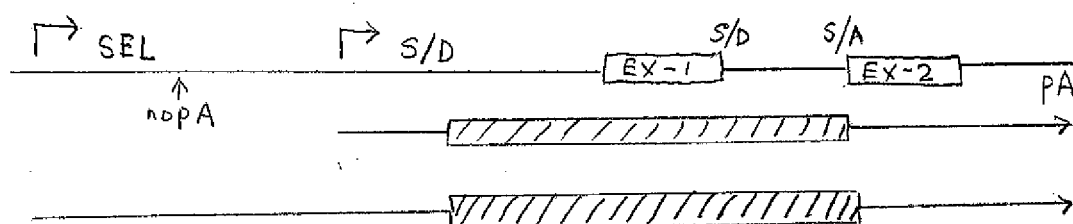


Fig. C.

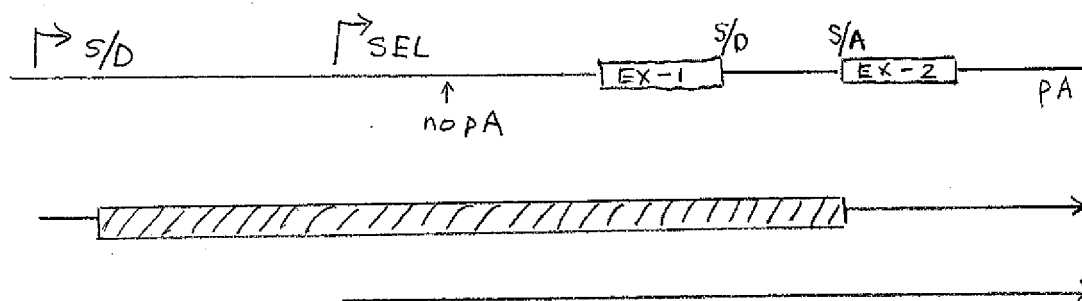





Fig. D.

 spliced out of transcript

 transcript

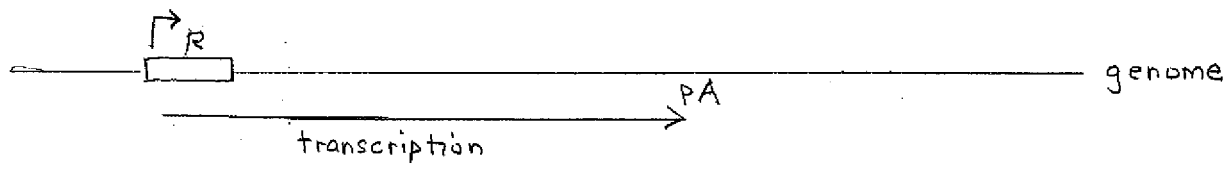
PA poly A

S/D splice donor

 promoter

SEL selectable marker

Attachment B



vector

R = reporter

$\overrightarrow{\text{P}}$ = promoter

pA = poly A

An Efficient Gene-Trap Method Using Poly A Trap Vectors and Characterization of Gene-Trap Events¹

Hitoshi Niwa, Kimi Araki,² Shigemi Kimura, Shin-ichi Taniguchi,³ Shoji Wakasugi, and Ken-ichi Yamamura

Department of Developmental Genetics, Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto, Kumamoto 862

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New trap vectors (U1 and U2) have been developed to trap genes in murine embryonic stem (ES) cells. The polyA addition signal of the neomycin phosphotransferase II (*neo*) gene was removed from these vectors so that they needed to trap an endogenous polyA signal for expression of the *neo* gene. The frequency of gene-trap events of these vectors was about five times higher than with the vector containing the polyA signal, and only one copy of the trap vector was integrated in most cases. Four out of five 5'-flanking regions of the integrated vector in ES cell lines were found to be novel endogenous promoters, suggesting that this method is efficient for trapping genes in ES cells. In two cases analyzed, large deletions or rearrangements spanning more than 10 kb were found in the 3'-flanking region of the trap vector introduced by electroporation. This result suggests that phenotypes observed in homozygotes with a mutated allele could be due to the disruption of a gene adjacent to the trapped gene, but not of the trapped gene.

One strategy for monitoring transcriptionally active regions of a genome involves use of an enhancer trap, based on the fact that transcription of a reporter gene containing a minimum promoter is activated by cellular enhancers. In *Drosophila*, the enhancer trap strategy has been used successfully in large-scale screening of developmentally regulated genes (1, 2). The β -galactosidase (*lacZ*) reporter gene provided a sensitive and easily assayable gene product to detect expression in whole embryos. A similar strategy was applied to mice, and transgenic mice carrying enhancer trap vectors were found to exhibit unique temporal and spatial patterns of *lacZ* expression (3, 4). To use this strategy effectively on a large scale in mice, *lacZ* reporter constructs were introduced into mouse embryonic stem (ES) cells. However, in mice the expression of a reporter gene is often influenced by the integration site, and an enhancer is sometimes located far from a coding region. These features make it difficult to identify and isolate the mouse endogenous gene. A second type of vector which was designed as a gene trap was developed to clone the promoter or exon sequences of the endogenous gene directly. The gene-trap vector contains a splice acceptor instead of a weak promoter in front of a *lacZ* gene (5). Thus, gene trap vectors are expected to generate spliced fusion transcripts between the reporter gene and the endogenous gene present at the site of integration (6, 7). In addition, all insertions of the gene trap vector may result in a mutation in the host

genes. Overall this gene-trap strategy in embryonic stem (ES) cells is a powerful method because it allows isolation of numerous unknown genes functioning in the early stages of development and morphogenesis, examination of the expression pattern of the endogenous gene, and the obtaining of insertional mutant mice (5, 6, 8, 9). This gene trap method in ES cells can thus be regarded as gene-targeting to unknown genes, as opposed to gene-targeting to known genes, which is known as homologous recombination. Previous reports have shown that the reporter constructs were integrated into endogenous loci, leading to specific patterns of expression during development and to disruption of the endogenous genes at the site of integration.

Although we can expect the 5'-flanking region of the trap vector to remain intact, the 3'-flanking region may be deleted or rearranged. This may make it difficult to clone the disrupted gene. Furthermore, it is difficult to deduce the connection between the disrupted gene and phenotype because a gene adjacent to the integration site may also be disrupted. Such deletions or rearrangements were not fully analyzed in the previous reports. In this study we examined the extent of deletions or rearrangements of the 3'-flanking regions of the gene-trap vectors. We also attempted to increase the frequency of trap events over random integrations and to select ES clones in which only a single gene is trapped or disrupted by the trap vector. The constructs used in our gene trap experiments consist of promoter-less *lacZ* and neomycin phosphotransferase (*neo*) expression unit without its own polyA addition signal sequence. We removed polyA addition signal sequence because the *neo* gene is expected to be expressed only when the trap vector is integrated in such a way as to utilize the polyA addition signal of the endogenous gene. The results demonstrate that a large deletion or rearrangement spanning more than 10 kb does occur during integration and that a gene-trap

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Present addresses: ² Department of Pathology, Centre Medical Universitaire, 1 Rue Michel Servet, CH1211, Geneva 4, Switzerland; and ³ First Department of Internal Medicine, Tottori University School of Medicine, Yonago, Tottori 683.

vector without the polyA addition signal at the 3'-end of the *neo* gene enriches trap events in ES cells.

MATERIALS AND METHODS

Construction of Plasmids—We first constructed pAcneoAn(−) and pAcneoAn(+) by replacing the *XhoI*-*MluI* sites of pMC1neo-polyA(−) and pMC1neo-polyA(+) (each purchased from Stratagene) with a 1.3-kb *XhoI*-*HindIII* fragment containing the chicken β -actin promoter region. Then, *Bam*HI sites of the pMC1neo were converted to unique *XbaI* sites by using a *XbaI* linker. A 230-bp *PvuII*-*MnII* fragment of CDM8 (10) containing the amber suppressor tRNA(*SupF*) gene was excised and the *MnII* site was converted to blunt end by a standard procedure (11). An *EcoRI*-*AatII* fragment of pXSP64, a derivative of pSP64 (Promega) in which the *XbaI* site was removed, was excised and both sites were converted to blunt ends. Plasmid pXSP-SupF was constructed by ligating these two fragments. DNA fragments used in the gene trap were as follows. (i) A 4.0-kb *XbaI*-*Bam*HI fragment excised from pAGS-*lacZ* (12) containing the rabbit β -globin splice acceptor sequence from the intron 1/exon 2 boundary, *lacZ*, and polyA addition signal sequence from SV40 largeT gene. The cohesive end of *Bam*HI was converted to a flush end by standard procedures. (ii) A 2.2-kb *SmaI*-*Bam*HI fragment from pXSP-SupF, in which the *Bam*HI end was converted to a *XhoI* site by using a *XhoI* linker. (iii) A 2.0-kb *XhoI*-*XbaI* fragment from pAcneoAn(−) for U2 or pAcneoAn(+) for U2pA. These fragments were ligated to produce U2 and U2pA. U1 was constructed by removing the *HindIII*-*KpnI* fragment containing the translation start codon of *lacZ* from U2.

Cells and Electroporation—ES MS-1 cells (derived from C57BL/6) were cultured using mitomycin-treated STO feeder cells in Dulbecco's Modified Eagle's Medium (Sigma) containing 15% fetal calf serum (Sera Lab.), 0.1 μ M 2-mercaptoethanol, 110 μ g/ml sodium pyruvate, 4.5 mg/ml D-glucose, and 1,000 units/ml recombinant murine

leukemia inhibitory factor (LIF). LIF was produced by transient expression of murine LIF cDNA using the vector pCAGGS-LIF (13) in BMT-10 cells (14). Murine LIF cDNA was generously provided by the Genetics Institute (Boston, U.S.A.). The cells were electroporated with the linearized DNA construct at 40 μ g/ml using a Bio-Rad Gene Pulser, and selected with G418 at 150 μ g/ml for 10 days. In the transfection experiment to test promoter activity, undifferentiated F9 embryonal carcinoma cell lines were used. These cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum and transfected by the calcium phosphate method. ES MS-1 cells were induced to differentiate by incubation in the presence of 100 nM retinoic acid for 4 days. Cells were fixed in 1% glutaraldehyde and stained with X-gal as described.

Production of ES Cell Chimeras—ES-D3 cells (derived from 129) (15) were cultured using LS-10 cells, subclones of STO cells, as feeder cells. C57BL/6 blastocysts were injected with 10 to 15 ES cells and transferred to the uterus of pseudopregnant ICR recipients as described previously (16). Chimeric males were mated with C57BL/6 mice and germline transmission was confirmed by the D3 agouti coat color and the inheritance of introduced trap vector DNA.

Cloning of the Flanking Mouse Genomic DNA—Genomic DNA flanking the integrated vector from each cell line was isolated by the plasmid rescue method. Each genomic DNA was digested with appropriate restriction enzymes and ligated at 10 μ g/ml to obtain circular molecules. These circularized DNAs were transformed into *E. coli* JM109 by electroporation and plated on LB/agar plates using an ampicillin drug selection for the plasmids. Rescued plasmids were analyzed by restriction mapping and DNA blotting, using standard procedures.

3'-RACE Analysis of *neo* Transcription—The RACE strategy with several modifications was used to amplify cDNA sequences 3' to *neo* gene (17). In brief, mRNA was reverse-transcribed using a dT17-adaptor primer, 5'-GAC-TCGAGTCGACATCGATTTTTTTT-TTTTTTTTTT-3'; and first amplification was subsequently performed using the adaptor primer, 5'-GACTCGAGTCGACATCGAT-3', and the first *neo*-specific primer, 5'-GCGTTGCTACCCG-TGATAT-3'. Then, second amplification was performed using the adaptor primer and second *neo*-specific primer, 5'-CCATCGATTTCGAGCGCATCGCCT-3'. Amplified cDNA was cloned into pBluescript KS(−) (Stratagene) for sequencing.

Nucleic Acid Hybridizations—DNA (10 μ g) digested with appropriate restriction enzymes was size-fractionated on a 0.7% agarose gel in Tris/borate buffer and transferred to a nylon membrane (HybondN+; Amersham). Hybridization of the blots with probes labeled by random-priming was carried out at 65°C in a mixture containing 5 \times SSPE, 5 \times Denhardt's solution, 0.5% SDS, and heat-denatured salmon sperm DNA (100 μ g/ml). Blots were washed in 2 \times SSC for 10 min at room temperature and then in 0.2 \times SSC/0.1% SDS for 50 min at 65°C, and signals were detected by use of BAS 2000 (Fuji).

RESULTS

Effects of Removal of the PolyA Addition Signal of *neo*—ES MS-1 cells were transfected by electroporation with

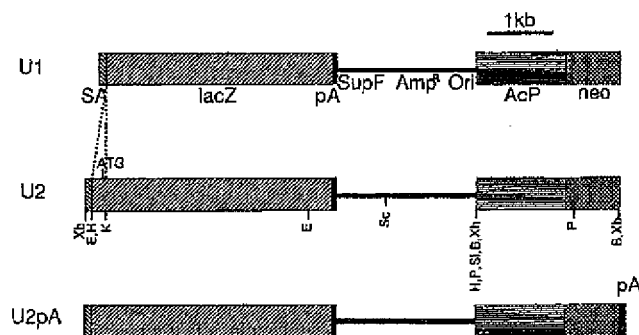


Fig. 1. Construction of gene-trap vectors U1, U2, and U2pA. U1 has neither the start codon of the reporter *lacZ* gene nor the polyA addition signal of the *neo* gene, U2 has only the start codon, and U3 has both. The restriction maps of each vector are shown. Abbreviations: SA, splice acceptor site; *lacZ*, β -galactosidase gene; pA, polyA addition signal; SupF, t-RNA gene suppressor mutation; Amp^r, ampicillin resistant gene; Ori, replication origin in *E. coli*; AcP, chicken β -actin promoter; *neo*, neomycin phosphotransferase II gene; ATG, initiation codon; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sca*I; Sl, *Sal*I; Xb, *Xba*I; Xh, *Xho*I. See "MATERIALS AND METHODS" for details.

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gene-trap vectors U1, U2, and U2pA. To examine the effect of the absence of the polyA addition signal sequence of the *neo* gene, we introduced U2 and U2pA plasmid DNA linearized with *Xba*I into ES cells by electroporation, selected with G418 for 10 days, and stained with X-gal. Of the total G418^r colonies, 7.7% stained blue when the polyA addition signal was removed, in contrast to 1.1% when the polyA addition signal was included (Table I-1). The result indicates that trapping of the endogenous polyA addition signal contributes to the enrichment of the gene trap by the reporter *lacZ* gene in the total G418^r colonies. When the polyA addition signal is removed from the *neo* gene, *lacZ* may be expressed under the control of the actin promoter of the *neo* gene, or the *neo* gene may be expressed using the polyA addition signal of the *lacZ* gene as a fusion transcript when more than 2 copies of the trap vector are integrated in tandem array. To estimate the frequency, trap vectors digested with *Sca*I were introduced, selected and stained. Of the total G418^r colonies, 1.3% in U2 and 0.7% in U2pA stained blue (Table I-1). On the other hand, ten times as many resistant colonies were obtained by the introduction of U2 linearized by *Sca*I rather than by *Xba*I. And the introduction of U2pA linearized by *Sca*I rather than *Xba*I produced three times as many colonies. These results suggest that the *neo-lacZ* fusion RNA was transcribed to produce fusion products with *neo* function but not with *lacZ* function, and that single-copy integration occurs in much higher frequency than multi-copy integration. In the case of U2pA, the polyA addition signal of the *neo* gene was located 43 bp upstream from the *Xba*I site and was far from the *Sca*I site. Thus, the polyA addition signal could be deleted during integration into the host genome, leading to the

reduction of colonies obtained by the introduction of U2pA.

Effect of the Presence or Absence of the Initiation Codon in the *lacZ* Gene—The U1 and U2 linearized by *Xba*I were introduced into ES cells and selected. There were three stop codons that fit in all reading frames in the region up to 30 bp upstream of the translation start codon of *lacZ* gene in U2 (Fig. 4A). Consequently all translation from fusion transcripts are expected to terminate at these stop codons and be reinitiated from the start codon of the *lacZ* gene, because such termination-reinitiation was previously reported to occur in mammalian cells (18). As the 144-bp region containing these stop codons and the start codon was excised from U2 to construct U1, the translation of *lacZ* occurs only when the *lacZ* gene is fused in-frame to the endogenous gene. In the intron, there are no stop codons and two ATG codons in the same frame. But these are out-frame to the *lacZ*. Thus, the *lacZ* is expressed as the fusion product when the *lacZ* is directly fused in-frame to an endogenous gene. Of the total G418^r colonies, 16.1% in U1 and 12.5% in U2 stained blue (Table I-2). When ES-D3 cells were used, 8.3% (2/24) and 33% (14/42) of the total G418^r colonies stained blue in U1 and U2, respectively. This suggests that there was no significant difference in the efficiency of gene trapping between U1 and U2, and that any variation of efficiency in these experiments was due to differences in experimental conditions for electroporation, as reported in previous gene-targeting experiments.

Cloning of Mouse DNA Flanking the Trap Vectors—We used sixteen trap lines obtained by the above experiments using U1 and U2 in the following analyses. Southern blots analysis of DNA from each cell line digested with *Bam*HI and *Pst*I showed that the vector was inserted into single site in all sixteen lines. To determine the copy numbers integrated, PCR analysis was done using a set of primers near the 5'- and 3'-ends of linearized vectors. No amplified DNA fragment appeared in fifteen of sixteen lines suggesting that a single copy of the vector was integrated into the

TABLE I. Expression of reporter gene in the transfected ES cells. 1×10^6 ES cells were electroporated with 32 μ g of linearized vectors, selected in 150 μ g/ml of G418 and stained with X-gal after selection for 10 days.

Expt. No.	Transfected vector	Linearized site	No. of G418 ^r colonies	No. of X-gal+ colonies
1	U2	<i>Xba</i> I	117	9 (7.7%)
		<i>Sca</i> I	1188	15 (1.3%)
	U2pA	<i>Xba</i> I	585	3 (1.1%)
		<i>Sca</i> I	1635	12 (0.7%)
2	U1	<i>Xba</i> I	68	11 (16.1%)
	U2	<i>Xba</i> I	48	6 (12.5%)

TABLE II. Characterization of rescued 5'-flanking region.

Clone	Vector type	Expression of <i>lacZ</i> ^a		Rescued 5'-flanking region	
		Undif.	Diff. ^b	Length (kb)	Promoter activity ^c
AYU-1	U2	+++	—	2.1	+
AYU-2	U2	++	+	1.2	+
AYU-3	U1	—	+	3.0	—
AYU-4	U1	+	++	N.D.	N.D.
AYU-5	U2	+	—	0.6	+
AYU-6	U2	+	—	0.6	+

^aExpression of the introduced reporter *lacZ* gene was determined by staining with X-gal for 12 h. The signs indicate: —, no staining; + to +++, increasing area of staining intensity. ^bEach gene trap ES cell line was stained before and after differentiation by retinoic acid. ^cEach rescued plasmid containing the 5'-flanking genomic region and the entire *lacZ* gene was introduced into F9-tk⁺ cells. After 3 days these transformants were stained with X-gal to determine the promoter activity of the rescued region. A plus sign indicates the presence of staining cells, a minus sign the absence of staining cells.

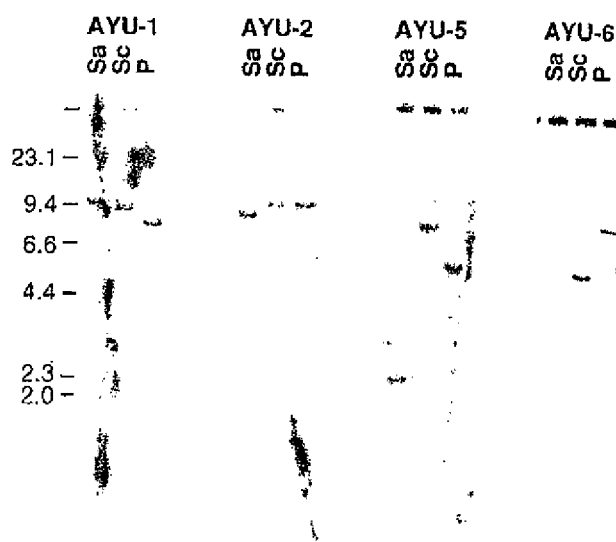


Fig. 2. Southern blot analysis of the genome of four trapped ES cell lines. Genomic DNAs (10 μ g) from each line were digested with *Sac*I, *Sca*I, or *Pst*I, run on agarose gels, blotted, and hybridized to *lacZ* probe. A single band was detected in each case, so only a single copy of the vector was integrated into the unique site of the genome.

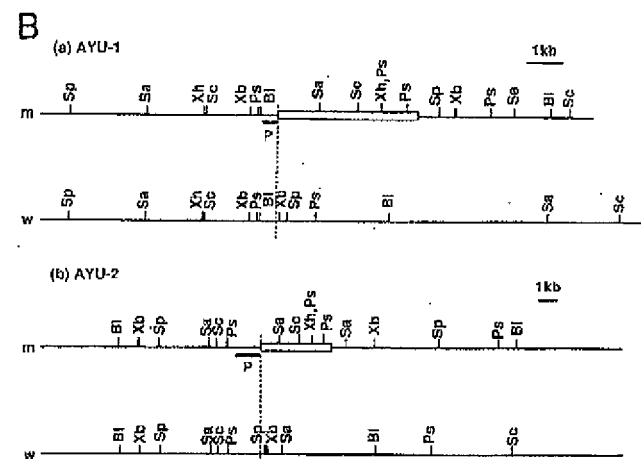
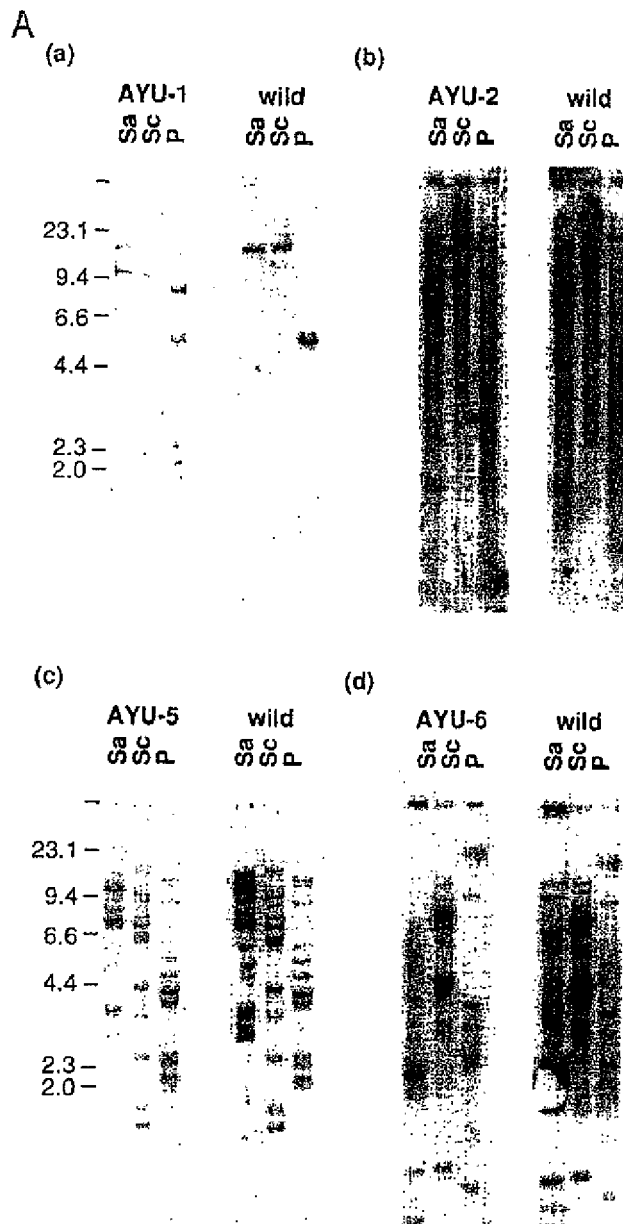


Fig. 3. (A) Southern blot analysis of the four trapped ES clones and normal ES cells. Genomic DNAs (10 μ g) were digested with *SacI*, *ScaI*, and *PstI*, run on agarose gels, blotted, and hybridized with each rescued genomic fragment flanking the 5'-ends of the integrated vectors. With AYU-1 and AYU-2 probes, only single bands were detected in wild-type ES cells and new additional bands associated with the gene-trap insertion in trap lines. Multiple bands were detected in both wild-type and trapped ES cells with AYU-5 and AYU-6. (B) Restriction maps of the AYU-1 and AYU-2 loci in the wild and mutant alleles. All restriction sites were determined by Southern blot analysis of the genomic DNA from each trapped ES cell line and wild-type ES cells. Abbreviations: BlnI, *BlnI*; Ps, *PstI*; Sa, *SacI*; Sc, *ScaI*; Sp, *SpeI*; Xb, *XbaI*; Xh, *XhoI*. The solid bars show the position of probes used in these analyses.

genome in these lines (data not shown). We rescued a genomic DNA fragment spanning about 600 to 3,000 bp flanking the 5'-end of the inserted vector by the plasmid rescue method from five cell lines, named AYU-1, AYU-2, AYU-3, AYU-5, and AYU-6, that showed expression of *lacZ* before or after induction of differentiation (Table II). Subcellular localization of X-gal staining was cytoplasmic (diffuse) in all six cases. The rescue failed in one line, AYU-4, in which many copies of the vector were integrated.

Identification of Cellular Promoters in Flanking Regions—The plasmid rescue method allows isolation of flanking regions connected to intact reporter genes, and the promoter activities of these regions can readily be estimated by re-introducing the rescued plasmid into appropriate cell lines and detecting transient expression of reporter

genes. We introduced plasmids rescued from five lines into undifferentiated F9 cells by the calcium phosphate method and stained these transfectants with X-gal after 3 days. Four of the five lines showed expression of reporter genes (Table II), suggesting that promoter elements were retained in rescued fragments and these were sufficient for expression in F9 cells. These four flanking regions were used to probe Southern blots of DNA from each of the four cell lines and non-transfected ES MS-1 cells. In the case of AYU-1 and AYU-2, these probes detected only one or two bands in normal ES MS-1 DNA and appropriate additional bands in each cell line DNA, suggesting that these DNA fragments were derived from cellular genes. On the other hand, the probes of AYU-5 and AYU-6 detected more than ten bands in normal DNA. This suggests that they were derived from gene families or contain repetitive sequences such as retrotransposon. These four flanking genomic DNAs were sequenced and searched for consensus promoter motifs, and in all cases typical promoter motifs were found. In the flanking region of AYU-1, a typical TATA box, an NF-1 binding motif containing inverted repeats, an Sp-1 binding site, and two typical octamer binding sites were observed. We are most interested in this clone because of the motifs in this region and the strong expression of the reporter gene in the undifferentiated state followed by dramatic reduction of expression after differentiation. We are analyzing this in more detail. The sequence of this region will be reported separately. In the AYU-2 flanking region, there are two GC boxes, GGGCGG, at only 50-bp upstream from the integration site, and the AYU-6 flanking region also contains GC box-like motifs

Efficient Gene Trap Using PolyA Trap Method

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(A) β -globin/*lacZ* junction

ctctagagcctctgctaaccatgttcacgttccttcttttctctacagCTCCTGGGCA
 ACGTCTGTTGTTGTGCTGTCATCATTTTGGCAAGAAATTCAGCTGGGATCTC
 TATAATCTCGCGCAACCTATTTCCTTCGACACTTTTAAAGCCGTAGATAACAGGC
 TGGGACACTTCACATGAGCGAAAAATACATCGTCACCTGGGACATGTTGCAGATCCATG
 CACGTAACTCGCAAGCCGACTGATGCTTCTGAACAATGGAAAGGCATTATTGCGGTA
 AGCCGTGGCGGTCTCTGAGCGGTGGGTGAAGACCA
 KpnI

(B) AYU-2

TTGTGCTGTGTGCTGCGCGCGATGTTCCGTACGACCTCGATGGCTGATTAGCGCTAA
 GCTGCGCTACTGTTAGTTGAAACACGTTAGACGAAACGAGGTCTCTGCTGTGCTGC
 TGAASATCTGGAGTCACCCCTTCTGCAGGACCTGGGATTGAGCGGGCGCTCCCTCAT
 CTCGGCAGCCTTCCTGCTTACGCGCCACCCCACTTTTTCCTTCCTACGAC
 CTGCGTCTGCGTCCACCCCGACCGCTgctaaccatgttcacgttccttcttttctct
 acag

Fig. 4. Nucleotide sequence of the gene-trap splice acceptor and the rescued endogenous promoter regions from three gene-trap cell lines. (A) Nucleotide sequence of the β -globin/*lacZ* junction region of the U2 gene-trap vector. The intron region of β -globin containing the splice acceptor site is shown in lowercase letters and other sequences in uppercase letters. The *lacZ*-coding sequence was fused to the second exon of β -globin, 40 codons long, derived from *E. coli gpt* gene, which contains prokaryotic promoter and 28 codons from *E. coli trpS* gene. The initiation codon of *lacZ* is boxed, and three stop codons between the splice acceptor and the initiation codon are underlined. In U1, the sequence between the two arrows in the figure is deleted. Sequences of 5'-flanking genomic regions and junctions of integrated vectors rescued from the AYU-2 (B), AYU-5 (C), and AYU-6 (D) cell lines are shown. Partial sequence of the AYU-2 clone, 1.1 kb in length, and entire sequences of AYU-5 and AYU-6 are shown. The β -globin sequence of the trap vector is shown in lowercase letters and the genomic sequence in uppercase letters. Predicted TATA-box sequence is boxed and GC box-like sequences are underlined.

(C) AYU-5

CTGCAGCACACTCCCTTTTCTTTGGTCTGTGACCTAGCTCTTGTCTGCAGTGTTTTAC
 CTGACAGTATACAGTAAGCTGTTCCTCTGGTTCTCATCTGCCAAGTATATTTCCACAC
 ACAAAACCCCTGGCACAAGGTTTTTCCAGATGCTCCAGTAAATTTATTCATAG
 GCTACAGCGGCTCCAAAGGAGAGACTAGGTCCACAAGCAGCACAGATGGCCCCCTA
 TGGCTGGCTGACGCTTACCATCCATGCGCTGCAAGACCTACCACACAGTCTATTCTTG
 GTAAGAAGCTTGTCTCCCGAGGCTTTATGGGAGATGACTTCTACTTACCATGTCTGAAG
 TTCTGATCTTGGCTGATTACCCCTCCAGCACTCACTTAAGACACCTAAGAAAGCAACA
 CATAGAAAGACTCAGGTCACTGAGTTAAAGAGAGGAACCAAGGGCTTAGTACCCGGA
 AGAGCCACCTCTCTTCTGACTGGCAGCTCCGTGTGAGTTTCTGATTGGCTACTGAGT
 CTTGAATGACATgagcctctgctaaccatgttcacgttccttcttttctctacag

(D) AYU-6

CTGCAGTCCACCTTCAGATAAATTCCTACTTGGAGTGCCTTACTATCTCTAAGCTTTAG
 TAAATCCTTTACATCATGAATCTGCACTTTTATATCACAGCTTAATATTGGATTGCTA
 CCCAGCAGATGAGATGCTTTTACAACCTCCACAAAATTAACCTCAAAATGGATTGTCCA
 CCTTAAGCTCAAATGCTAAGAACAGAACGTGTAGAACTAGGACTCTCTCAGTTTGGGA
 TCCTGACAAAGATCTTTTACACACAGCCCAAAATGCTCTGCACAAAGATGACGCCCCCA
 CAGTGAGGTTATTAGAGGCTACAGGTGGGTCCCTAGTGGCTAGGGCTCAGGGGACC
 TCACACGTTGGGCGGCTTCCAGTCACTCCCGGAAAGACCCACACACAGTTCGCGC
 TGGTCAGACCCCTGCTTCTCCCATGTTGGCAGTACCCTGTGCTCACCATGTGCGAAC
 TTCGGAGCTTGCTGAGCACAGTCCACAGAATCCAGCGGAGGACAGGAGCAGACAC
 ACAGGCCCATCAAGCTGCTCAGCGACTAATTGAATTCGCGCAAGGCATCCGGAAA
 AACCCGCTTCCCTTCTGACTGGCAGGTGACCTAAAGGCTCTGATTGGCCAATAAGTC
 tgctaaccatgttcacgttccttcttttctctacag

near the integration site. In AYU-5, a TATA box and GC box-like motifs are found in the middle of the rescued 600-bp DNA fragment. A search of the GenBank data base (Version 18) for these four nucleotide sequences indicated that these sequences were novel. In AYU-1 and AYU-2, we also cloned 3'-flanking regions by the plasmid rescue method after digestion with *EcoRI*.

Transcription of the Rescued Sequences—In Northern blot analysis using these 5' rescued regions as probes, the AYU-3 and AYU-5 flanking regions detected endogenous transcripts. In AYU-3, transcripts of approximately 1.8-kb were detected in almost all adult tissues; and in AYU-6, 2.5-kb transcripts were detected only in undifferentiated ES cells (data not shown). In AYU-1, transcripts of about 10-kb were detected in adult brain, duodenum, and spleen, and small 8-kb transcripts were detected in testis using the 3'-rescued region as a probe. In AYU-2, 5-kb transcripts were detected in almost all adult tissues (data not shown). The characteristic sequence of the promoter region and the ubiquitous expression of endogenous transcripts suggests that the trap vector may be integrated into a housekeeping gene in AYU-2.

Rearrangement and Deletion of Mouse Genome Near the Integration Sites—The expression of reporter gene in the trap vector means that the 5'-flanking region remains intact. However, deletions or rearrangements in the 3'-flanking region were not examined in previous reports. This is important because phenotypic consequences could be due to the disruption of other gene(s) located in the 3'-flanking region of the trapped gene or in another region rearranged during the integration of the trap vector. We addressed this question by examining the 3'-flanking region of AYU-1 and AYU-2. In the case of AYU-1 and AYU-2, the rescued genomic DNA fragments detected single bands on genomic Southern blots in normal ES MS-1 cells and additional unique bands in each respective trap line (Fig. 3A), and the *lacZ* and *neo* probes also detected single bands in each respective trap line (Fig. 2). Thus, we made restriction maps of wild and mutated alleles near the integration sites (Fig. 3B) and compared them with each other. The results suggest that there were large deletions or rearrangements extending over more than 10 kb around the integration sites in these two cases. In contrast, deletions of only 2 to 20 bp were found in the 5'- or 3'-ends of the introduced vectors in all six cases, as revealed by sequencing of each rescued plasmid (Figs. 4 and 5).

PolyA Signal of *neo* Transcript—As stated earlier, the frequency of gene-trap events is higher in the trap vector without the polyA signal of the *neo* gene than in that with it. To examine whether this is due to the utilization of the

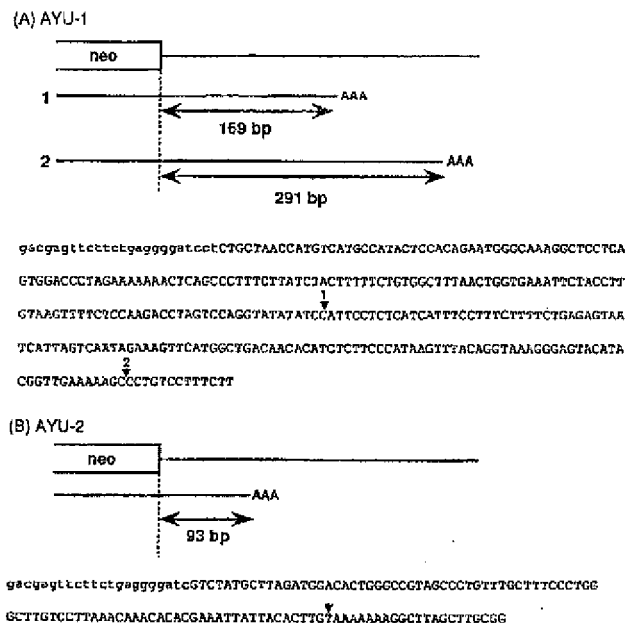


Fig. 5. Nucleotide sequence of the fusion transcripts of the *neo* genes lacking their own polyA addition signal. The genomic regions downstream of the 3'-ends of the integrated vectors are cloned from AYU-1(A) and AYU-2(B) cell line and sequenced partially. The regions of the *neo* gene are shown in lowercase letters and the flanking genomic sequence in uppercase letters. The polyA addition sites found in cDNA of the fusion transcripts cloned by the 3'-RACE procedure are marked by a vertical arrow. In the case of AYU-1, two cDNA fragments of different length, 169 and 291 bp, were isolated. In these clones the polyA tail is added at different sites of the genomic region, suggesting that each transcript may use different genomic sequences as the polyA addition signal. No completely matched sequences to the consensus of the mammalian polyA addition signal were found in upstream regions of polyA addition sites in these cases.

polyA signal of an endogenous gene, we cloned partial cDNA of *neo* transcripts by the 3'-RACE method from five trap clones (AYU-1, AYU-2, AYU-4, AYU-5, and AYU-6). In the agarose gel electrophoresis of the products from second cycle PCR, we detected two bands of approximately 350 and 250 bp in AYU-1, two bands of approximately 330 and 310 bp in AYU-2, and one band of approximately 300 bp in AYU-5, and these bands were recovered, digested with *Cla*I, and subcloned into the *Cla*I site of pBluescript KS(-) for sequencing. Sequence analysis revealed that three of the five clones had the identical sequence to the 3'-end of the *neo* gene at one end and polyA at the other end, and unknown sequence between them. The unknown sequences of two *neo* transcripts in AYU-1 were identical to the 3'-flanking genomic region of the integrated vector of AYU-1 cloned by the plasmid rescue method. So these transcripts were read through the *neo* gene and 3'-flanking genome, but there were no clear sequences completely matching the consensus sequence of the mammalian polyA addition signal, AATAAA, in this genomic region. Some similar sequences were found in this region but we could not conclude which sequence functions as the polyA addition of the *neo* transcript. We obtained the same result in the case of AYU-2. No definite band appeared in AYU-4 and 6; and in these cases, the *neo* gene may be transcribed through the 3'-flanking genome or fused to an exon of endogenous gene

by splicing, leading to the production of long mRNA of which we could not amplify the 3'-region by the 3'-RACE method.

Germ-Line Transmission of Genes Trapped by PolyA Trap Vectors—Unfortunately the trap clones derived from ES MS-1 cells could not contribute to germ cells in chimeric mice. We also carried out the gene-trap experiment using the ES D3 cells. In this experiment, the cell lines established from the clones expressing the reporter genes in undifferentiated states were injected into C57BL/6 blastocysts and chimeric mice were produced. Nine out of 12 chimeric mice showed germ-line transmission. These offspring are now breeding to homozygosity to test whether any phenotype will appear.

DISCUSSION

The selective disruption of genes expressed in undifferentiated embryonic stem cells has been reported (5, 6, 8, 9). However, isolation of developmentally regulated genes that are silent in the undifferentiated state remains a formidable task. To avoid laborious and time-consuming work, we developed a new method to increase the gene-trap frequency by removing the polyA signal from the gene-trap vector.

In previous reports of the gene trap using ES cells, deletions or rearrangements near the integration sites were not fully characterized, despite their particular importance in considering the relationship between the rearrangements and the mutated phenotypes. In two cases we demonstrated the existence of large deletions and/or rearrangements in the genome around the integration sites. Under such circumstances we could not conclude that abnormal phenotypes are due to the disruption of the trapped gene, because a gene adjacent to the trapped gene may also be disrupted. To solve this problem, it is necessary to use other methods of vector introduction or to construct a new vector. Retrovirus vector is shown to introduce only a single copy of the exogenous DNA into the host genome with small risk of DNA rearrangement. However, it is often laborious to obtain a high titer of recombinant retroviral vectors. Thus, we tried to develop a new trap vector which is designed to trap a polyA signal in addition to a promoter or an exon. By deleting the polyA signal from the *neo* gene, we can expect to select trap clones with minimal deletions or rearrangements and to enrich the trap events over random integrations. However, two problems may arise by using this vector. One is the possibility of expression of the *neo* gene by use of the polyA signal of the *lacZ* gene; the other is the possibility of expression of the *lacZ* gene under the actin promoter of the *neo* gene when more than two copies of the trap vector are integrated. The results suggest that the former could occur but that the latter did not. As only one copy of the trap vector is integrated in most cases, it is not necessary to take into consideration the expression of the *neo* gene by use of the polyA signal of the *lacZ* gene. In any case, an approximately fivefold enrichment of the trap events over random integrations was observed with our trap method, although deletions or rearrangements spanning more than 10 kb still existed.

Skarnes *et al.* demonstrated that the splice acceptor site upstream of the reporter gene was used properly and

efficiently to create fusion gene products (6). But this was not found in our experiment, due to the loss of a consensus sequence required for proper splicing from the vector upon integration into the host genome, as confirmed by sequence analysis of the integrated vectors. It was found that a short sequence of from 2 to 20 bp was deleted from the ends of the integrated vectors and that the deleted region often contained the splice acceptor consensus sequence. Thus, it is necessary to add a longer sequence in front of the splice acceptor to retain the function. This small deletion may be the reason for integration of the trap vector downstream of the promoter region rather than the exon. In four out of five trap clones analyzed, trap vectors were integrated just downstream to the novel endogenous promoters. Macleod *et al.* reported a similar phenomenon with a promoterless *neo* gene (19). In this case, most insertions were shown to be in close proximity to CpG islands. This is consistent with our results. They also showed short regions of homology between the genomic target DNA and the construct ends, although we could not confirm this. Retrovirus vectors were also shown to have a tendency to be integrated close to the DNase I hypersensitive site resulting in the promoter trap (20, 21). The advantage of the promoter trap will be the creation of null alleles in most cases, because it is unlikely that a fusion protein will retain much of its original activity, and expression of the normal message from the tagged gene could only arise if there is alternative splicing bypassing the promoter trap.

The advantage of the polyA trap method in gene-trap experiments will become apparent when it is combined with an appropriate *in vitro* differentiation system, directed to specific pathways. With such a combination, we should be able to prescreen clones of interest and reduce the amount of work required for generation of chimeric mice using every ES clone. For example, the ES cells spontaneously start to differentiate in suspension culture in the absence of the leukemia inhibitory factor (LIF) and form an embryoid body resembling an early post-implantation embryo (15). At least the visceral endoderm, the primitive ectoderm and the mesodermal precursors are differentiated in this system; so that we can analyze the genes involved in the differentiation of these cell lineages.

Systematic isolation of developmental control genes *via* gene-trap events will greatly facilitate molecular understanding of mammalian development.

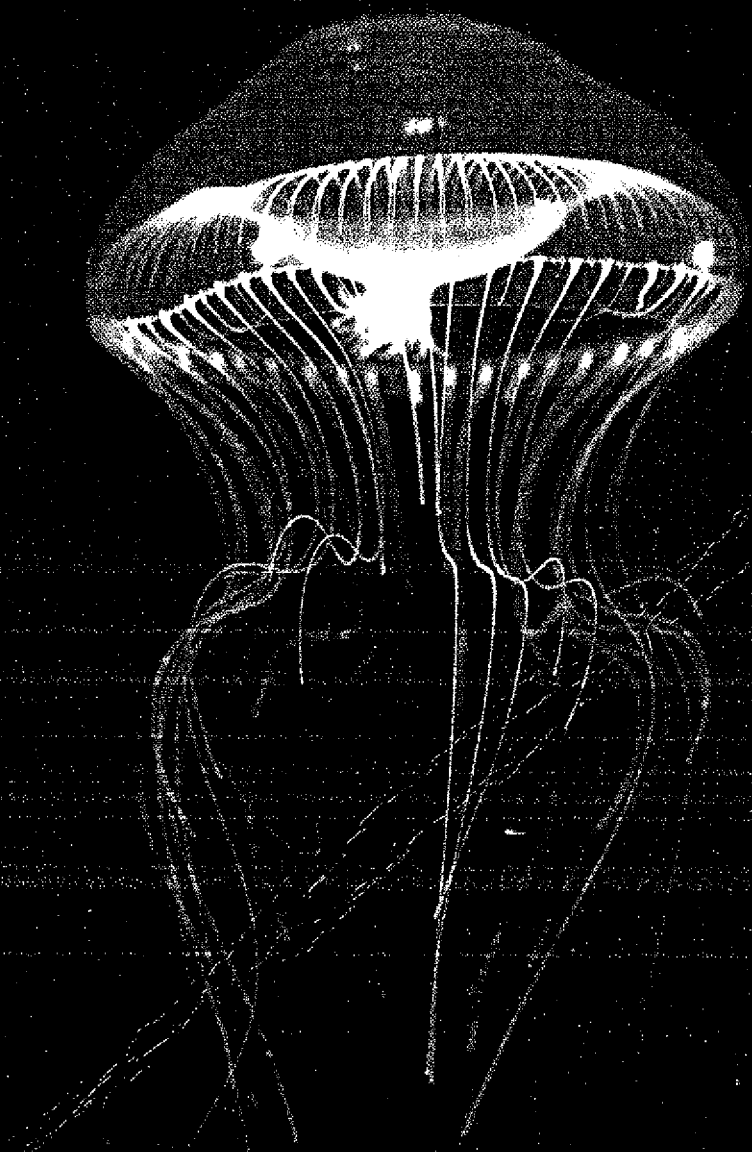
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Joseph Sambrook

PETER MACCALLUM CANCER INSTITUTE AND THE UNIVERSITY OF MELBOURNE, AUSTRALIA

David W. Russell

UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER, DALLAS



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Chapter 16

Introducing Cloned Genes into Cultured Mammalian Cells

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| Chloroquine Diphosphate | 16.53 |
| Electroporation | 16.54 |

A VARIETY OF STRATEGIES ARE AVAILABLE FOR THE DELIVERY of genes into eukaryotic cells. These techniques fall into three categories: transfection by biochemical methods, transfection by physical methods, and virus-mediated transduction. This chapter deals with the first two categories; the third approach is covered extensively in Section 8 of *Cells: A Laboratory Manual* (Spector et al. 1998b). The choice of a particular transfection method is determined by the experimental goal (e.g., the type of assay to be used for screening, the ability of the cell line to survive the stress of transfection, and the efficiency required of the system).

Biochemical methods of transfection, including calcium-phosphate-mediated and diethylaminoethyl (DEAE)-dextran-mediated transfection, have been used for nearly 30 years to deliver nucleic acids into cultured cells. The work of Graham and van der Eb (1973) on transformation of mammalian cells by viral DNAs in the presence of calcium phosphate laid the foundation for the biochemical transformation of genetically marked mouse cells by cloned DNAs (Maitland and McDougall 1977; Wigler et al. 1977), for the transient expression of cloned genes in a variety of mammalian cells (e.g., please see Gorman et al. 1983b), and for the isolation and identification of cellular oncogenes, tumor-suppressing genes, and other single-copy mammalian genes (e.g., please see Wigler et al. 1978; Perucho and Wigler 1981; Weinberg 1985; Friend et al. 1988). More recently, a collection of cationic lipid (liposome) reagents has been used successfully for gene delivery into a wider range of cell types. In all three of these chemical methods (calcium phosphate, DEAE-dextran, and cationic lipids), the chemical agent forms a complex with the DNA that facilitates its uptake into cells.

Two physical methods of transfection are in common use: Biolistic particle delivery and direct microinjection work by perforation of the cell membrane and subsequent delivery of the DNA into the cell; electroporation uses brief electrical pulses to create transient pores in the plasmid membrane through which nucleic acids enter.

TRANSIENT VS. STABLE TRANSFECTION

Two different approaches are used to transfer DNA into eukaryotic cells: transient transfection and stable transfection. In transient transfection, recombinant DNA is introduced into a recipient cell line in order to obtain a temporary but high level of expression of the target gene. The transfected DNA does not necessarily become integrated into the host chromosome. Transient transfection is the method of choice when a large number of samples are to be analyzed within a short period of time. Typically, the cells are harvested between 1 and 4 days after transfection, and the resulting lysates are assayed for expression of the target gene.

Stable or permanent transfection is used to establish clonal cell lines in which the transfected target gene is integrated into chromosomal DNA, from where it directs the synthesis of moderate amounts of the target protein. In general (depending on the cell types), the formation of stably transfected cells occurs with an efficiency that is one to two orders of magnitude lower than the efficiency of transient transfection. Isolation of the rare stable transformant from a background of nontransfected cells is facilitated by the use of a selectable genetic marker. The marker may be present on the recombinant plasmid carrying the target gene, or it may be carried on a separate vector and introduced with the recombinant plasmid into the desired cell line by cotransfection (for further details, please see the information panels on **COTRANSFORMATION** and **SELECTIVE AGENTS FOR STABLE TRANSFORMATION**). In general, all of the methods described below are suitable for use in transient transfection assays, and all, with the exception of DEAE-dextran, may be used for stable transfection.

TRANSFECTION METHODS

Until recently, cloned DNA has been introduced into cultured eukaryotic cells chiefly by biochemical methods. During the past 10 years, the range of cell types that can be transfected efficiently has been extended with the development of liposome methods, which work well with suspension cultures, and with the use of physical methods such as electroporation and biolistic particle delivery, which may be used successfully with many cell lines that are resistant to transfection by other means. A brief summary of transfection methods is given in Table 16-1.

TABLE 16-1 Transfection Methods

METHOD	EXPRESSION		CELL TOXICITY	CELL TYPES	COMMENTS
	TRANSIENT	STABLE			
Lipid-mediated Protocol 1	yes	yes	varies	adherent cells, primary cell lines, suspension cultures	Cationic lipids are used to create artificial membrane vesicles (liposomes) that bind DNA molecules. The resulting stable cationic complexes adhere to and fuse with the negatively charged cell membrane (Felgner et al. 1987; Felgner et al. 1994).
Calcium-phosphate-mediated Protocols 2 and 3	yes	yes	no	adherent cells (CHO, 293); suspension cultures	Calcium phosphate forms an insoluble coprecipitate with DNA, which attaches to the cell surface and is absorbed by endocytosis (Graham and van der Eb 1973).
DEAE-dextran-mediated Protocol 4	yes	no	yes	BSC-1, CV-1, and COS	Positively charged DEAE-dextran binds to negatively charged phosphate groups of DNA, forming aggregates that bind to the negatively charged plasma membrane. Uptake into the cell is believed to be mediated by endocytosis, which is potentiated by osmotic shock (Vaheri and Pagano 1965).
Electroporation Protocol 5	yes	yes	no	many	Application of brief high-voltage electric pulses to a variety of mammalian and plant cells leads to the formation of nanometer-sized pores in the plasma membrane (Neumann et al. 1982; Zimmermann 1982). DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of the redistribution of membrane components that accompanies the closure of the pores. Electroporation can be extremely efficient and may be used for both transient and stable transfection.
Biolistics Protocol 6	yes	yes	no	primary cell lines; tissues, organs, plant cells	Small particles of tungsten or gold are used to bind DNA, in preparation for delivery into cells, tissues, or organelles by a particle accelerator system (Sanford et al. 1993). This process has been variously called the microprojectile bombardment method, the gene gun method, and the particle acceleration method. Biolistics is used chiefly to transform cell types that are impossible or very difficult to transform by other methods.
Polybrene Protocol 7	yes	yes	varies	CHO and keratinocyte	The polycation Polybrene allows the efficient and stable introduction of low-molecular-weight DNAs (e.g., plasmid DNAs) into cell lines that are relatively resistant to transfection by other methods (Kawai and Nishizawa 1984; Chaney et al. 1986; Aubin et al. 1997). The uptake of DNA is enhanced by osmotic shock and dimethylsulfoxide (DMSO), which may permeabilize the cell membrane.

TRANSFECTION CONTROLS

All transfection experiments should include controls to test individual reagents and plasmid DNA preparations, and to test for toxicity of the gene or construct being introduced.

Controls for Transient Expression

Negative Controls

In transient transfection experiments, one or two dishes of cells should be transfected with the carrier DNA and/or buffer used to dilute the test plasmid or gene. Typically, salmon sperm DNA or another inert carrier such as the vector used to construct the recombinant is transfected into adherent cells in the absence of the test gene. After transfection, the cultured cells should not detach from the dish nor become rounded and glassy in appearance.

Positive Controls

One or two dishes of cells are transfected with a plasmid encoding a readily assayed gene product such as chloramphenicol acetyl transferase, luciferase, *Escherichia coli* β -galactosidase, or green fluorescent protein, whose expression is driven by a pan-specific promoter such as the human cytomegalovirus immediate early gene region promoter and enhancer. Tracer plasmids of this kind are available from many different commercial suppliers who sell kits containing the enzymes and reagents needed for detection of the encoded protein. Because the endogenous levels of these reporter activities are typically low, the increase in enzyme activity provides a direct indication of the efficiency of the transfection and the quality of the reagents used for a particular experiment. This control is especially important when comparing results of transfection experiments carried out at different times. Cotransfecting the reporter plasmid with the test plasmid or genomic DNA also provides a control for nonspecific toxicity in the overall transfection process.

Controls for Stable Expression

Negative Controls

One or two dishes should be transfected with an inert nucleic acid such as salmon sperm DNA, in the absence of the selectable marker. After culturing for 2–3 weeks in the presence of the selected agent (G418, hygromycin, mycophenolic acid), no colonies should be visible.

Positive Controls

One or two dishes of cells should be transfected with the plasmid encoding the selectable marker in the absence of any other DNA. The number of viable colonies present at the end of the 2–3-week selection period is a measure of the efficiency of the transfection/selection process. A similar number of colonies should be present on dishes into which both the selectable marker and the test plasmid or gene were introduced. A marked discrepancy in the number of colonies on these two sets of dishes can be an indication of a toxic gene product (or in rare instances of a gene product that enhances survival of the transfected cells). If a particular cDNA or gene proves toxic to

recipient cells, consider the use of a regulated promoter such as metallothionein (a Zn^{2+} - or Cd^{2+} -responsive DNA), the mouse mammary tumor virus long terminal repeat promoter (a glucocorticoid-responsive DNA), a tetracycline-regulated promoter (Gossen and Bujard 1992; Gossen et al. 1995; Shockett et al. 1995), or an ecdysone-regulated system (No et al. 1996). Alternatively, conditional alleles of some genes can be constructed (Picard et al. 1988).

OPTIMIZATION AND SPECIAL CONSIDERATIONS

Irrespective of the method used to introduce DNA into cells, the efficiency of transient or stable transfection is determined largely by the cell type that is used (please see Table 16-1). Different lines of cultured cells vary by several orders of magnitude in their ability to take up and express exogenously added DNA. Furthermore, a method that works well for one type of cultured cell

TABLE 16-2 Commercial Kits and Reagents for Transfection

MANUFACTURER	WEBSITE ADDRESS	KIT/PRODUCT	METHOD OR REAGENTS
Amersham-Pharmacia Biotech	www.apbiotech.com	CellPfect Transfection Kit	$CaPO_4$ or DEAE-Dextran
Bio-Rad	www.biorad.com	CytoFectene Reagent	Cationic lipid
CLONTECH	www.clontech.com	CLONfectin Reagent CalPhos Mammalian Kit	Cationic lipid $CaPO_4$
5 Prime→3 Prime	www.5prime.com	Calcium Phosphate Transfection Kit	$CaPO_4$ DEAE-Dextran
Invitrogen	www.invitrogen.com	Perfect Lipid	Cationic lipid
Life Technologies	www.lifetech.com	Lipofectamine, Lipofectin, LipofectAce, Cellfectin Calcium Phosphate Transfection System	Cationic lipid (proprietary) $CaPO_4$
MBI Fermentas	www.fermentas.com	ExGen 500	Cationic polymer
Novagen	www.novagen.com		
Promega	www.promega.com	Transfast Transfection Tfx Reagents Transfectam ProFectin	Cationic lipid Cationic lipid Cationic lipid $CaPO_4$ or DEAE-Dextran
QIAGEN	www.qiagen.com	SuperFect Effectene Transfection Reagent Selector Kit	Activated dendrimer Nonliposomal lipid and DNA condensing agent Enhancer Both reagents
Quantum Biotechnologies	www.quantumbiotech.com	GeneSHUTTLE 20 and 40	Cationic lipid
Sigma Aldrich	www.sigma-aldrich.com	DEAE Dextran Kit Calcium Phosphate Transfection Escort, DOTAP, DOPE	DEAE-Dextran $CaPO_4$ Cationic lipid kits
Stratagene	www.stratagene.com	LipoTaxi MBS Mammalian Transfection Kit Mammalian Transfection Kit Primary ENHANCER Reagent	Liposome-mediated Modified $CaPO_4$ $CaPO_4$ and/or DEAE-Dextran Supplemented with lipid, $CaPO_4$
Wako Chemicals USA	www.wakousa.com	GeneTransfer HMG-1, -2 Mixture	Liposome-mediated

may be useless for another. Many of the protocols described in this chapter have been optimized for the standard lines of cultured cells. When using more exotic lines of cells, it is important to compare the efficiencies of several different methods. The protocols in this chapter present commonly used transfection techniques as well as methods that have proven successful with cell lines that are resistant to transfection by standard techniques. Commercial kits are available that provide collections of reagents for many types of transfections (please see Table 16-2).

Many techniques used in eukaryotic cell culture are not discussed in detail in this manual (for specific information on cell culture, please see Volume 1 of *Cells: A Laboratory Manual* [Spector et al. 1998a]). In particular, it is assumed that the conditions for optimal growth and passage of the cell lines to be used in this protocol have already been established.

The students study molecules now, spinning models across their computer screens and splicing the genes of one creature into those of another. The science of genetics is utterly changed... Sometimes I wonder where we have misplaced our lives.

Andrea Barrett
"The Behavior of the Hawkweeds."

Protocol 1

DNA Transfection Mediated by Lipofection

BECAUSE A LARGE NUMBER OF VARIABLES AFFECT THE EFFICIENCY of lipofection, we suggest that the conditions outlined in the following protocol be used as a starting point for systematic optimization of the system (for further details, please see the information panel on **LIPOFECTION** at the end of this chapter). Alternatively, a protocol recommended by a commercial manufacturer of a particular lipofectant can be used to begin the optimization process. Once a positive signal has been obtained with a plasmid carrying a standard reporter gene, each of the parameters discussed in the information panel on **LIPOFECTION** may be changed systematically to obtain the maximal ratio of signal to background and to minimize variability between replicate assays. From these results, optimal protocols can be developed to assay the expression of the genes of interest.

To explore the suitability of a wide variety of lipids for the task at hand, we recommend purchasing an optimization kit containing a series of individual lipids or combinations of lipids. Examples of such kits include the Tfx Reagents Transfection Trio (Promega), PerFect Lipid Transfection Kit (Invitrogen), and the Transfection Reagent Optimization System (Life Technologies).

The following protocol is a modification of a method provided by Mark Evans (Alexion Pharmaceuticals, New Haven, Connecticut).

MATERIALS

Buffers and Solutions

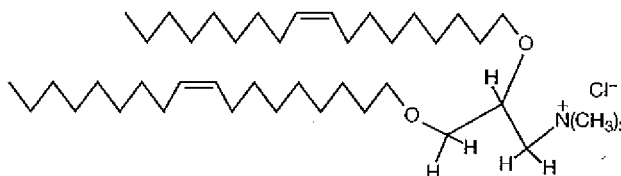
Please see Appendix 1 for components of stock solutions, buffers, and reagents.
Dilute stock solutions to the appropriate concentrations.

Lipofection reagent

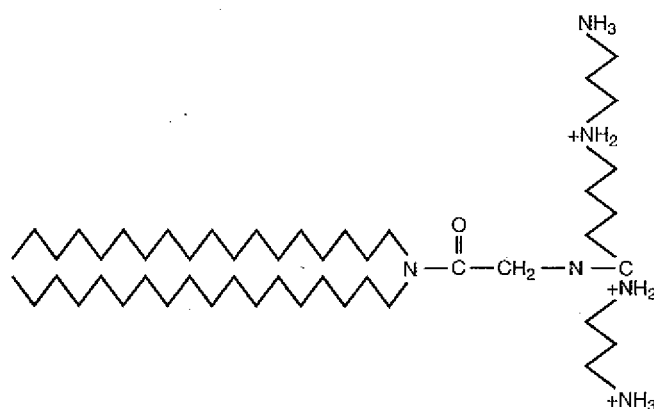
As illustrated in Table 16-4 and described in the information panel on **LIPOFECTION**, several types of lipofection reagents are available. This protocol describes the use of two common lipids:

- Lipofectin (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride [DOTMA]) (Figure 16-1). This monocationic lipid mixed with a helper lipid is usually purchased at a concentration of 1 mg/ml. DOTMA can also be synthesized with the help of an organic chemist (Felgner et al. 1987). If synthesized in-house, dissolve 10 mg each of dried DOTMA and the helper lipid dioleoyl phosphatidylethanolamine (DOPE, purchased from Sigma) in 2 ml of sterile deionized H₂O in a polystyrene tube (do not use polypropylene tubes). Sonicate the turbid solution to form liposomes before diluting to a final concentration of 1 mg/ml. Store the solution at 4°C.

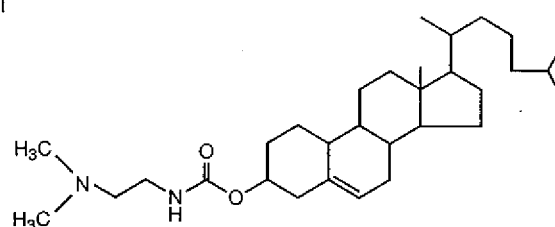
A. DOTMA



B. DOGS



C. DC-cholesterol



D. DOPE

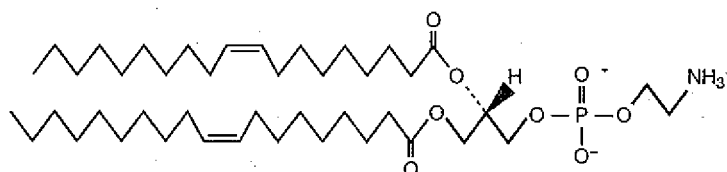


FIGURE 16-1 Structures of Lipids Used in Lipofection

For further descriptions of each of these lipids, please see Table 16-4.

TABLE 16-3 Dimensions of Dishes Used for Cell Culture

SIZE OF PLATE	GROWTH AREA (cm ²)	RELATIVE AREA ^a	RECOMMENDED VOLUME
96-well	0.32	0.04x	200 μ l
24-well	1.88	0.25x	500 μ l
12-well	3.83	0.5x	1.0 ml
6-well	9.4	1.2x	2.0 ml
35-mm	8.0	1.0x	2.0 ml
60-mm	21	2.6x	5.0 ml
10-cm	55	7x	10.0 ml
Flasks	25	3x	5.0 ml
	75	9x	12.0 ml

^aRelative area is expressed as a factor of the growth area of a 35-mm culture plate.

- Transfectam (Spermine-5-carboxy-glycinedioctadecyl-amide [DOGS]) (Figure 16-1). Polycationic lipids such as DOGS may be substituted for Lipofectin in the protocol. DOGS can be purchased and reconstituted as directed (Promega) or the lipid may be synthesized in-house to save money (Loeffler and Behr 1993). If synthesized in the laboratory, prepare a stock solution as follows: Dissolve 1 mg of polyamine in 40 μ l of 96% (v/v) ethanol for 5 minutes at room temperature with frequent vortexing. Add 360 μ l of sterile H₂O and store the solution at 4°C. Vortex the solution just before use. Polyamines, such as DOGS, do not require the use of polystyrene tubes; polypropylene tubes (i.e., standard microfuge tubes) can be safely used with these reagents. Polystyrene tubes must be used with DOTMA, because the lipid can bind nonspecifically to polypropylene.

NaCl (5 M) (optional)

Use as the diluent for DOGS.

Sodium citrate (pH 5.5, 20 mM) containing 150 mM NaCl (optional)

Use instead of sterile H₂O as the diluent for the plasmid DNA if DOGS is the lipofection reagent (Kichler et al. 1998).

Nucleic Acids and Oligonucleotides

Plasmid DNA

If carrying out lipofection for the first time or if using an unfamiliar cell line, obtain an expression plasmid encoding *E. coli* β -galactosidase or green fluorescent protein (please see the information panel on **GREEN FLUORESCENT PROTEIN** in Chapter 17). These can be purchased from several commercial manufacturers (e.g., pCMV-SPORT- β -gal, Life Technologies, or pEGFP-F, CLONTECH; please see Figures 16-2 and 16-3).

Purify closed circular plasmid DNAs by column chromatography or ethidium bromide-CsCl gradient centrifugation as described in Chapter 1. Dissolve the DNAs in H₂O at 1 μ g/ μ l.

Media

Cell culture growth medium (complete, serum-free, and [optional] selective)

Special Equipment

Test tubes, polystyrene or polypropylene

Polystyrene tubes must be used with DOTMA, because the lipid can bind nonspecifically to polypropylene.

Tissue culture dishes (60 mm)

This protocol is designed for cells grown in 60-mm culture dishes. If multiwell plates, flasks, or dishes of a different diameter are used, scale the cell density and reagent volumes accordingly. Please see Table 16-3.

Additional Reagents

Step 9 of this protocol may require the reagents listed in Chapter 17, Protocol 7.

Cells and Tissues

Exponentially growing cultures of mammalian cells

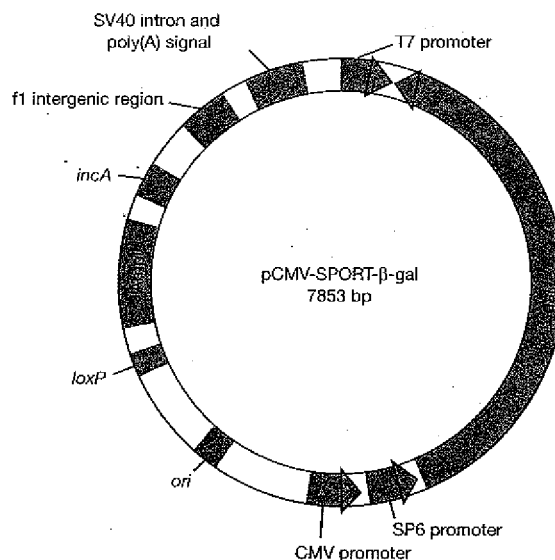


FIGURE 16-2 pCMV-SPORT-β-gal

pCMV-SPORT-β-gal is a reporter vector that may be used to monitor transfection efficiency. It carries the *E. coli* gene encoding β-galactosidase preceded by the CMV (cytomegalovirus) promoter that drives high levels of transcription in mammalian cells. The SV40 polyadenylation signal downstream from the β-galactosidase sequence directs proper processing of the mRNA in eukaryotic cells. (Reproduced, with permission, from Life Technologies, Inc.)

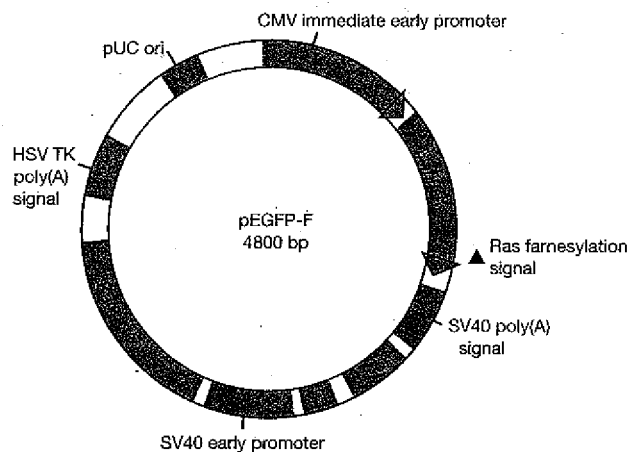


FIGURE 16-3 pEGFP-F

pEGFP-F is a reporter vector that may be used both to monitor transfection efficiency and as a cotransfection marker. The vector encodes a modified form of the green fluorescent protein, a farnesylated enhanced GFP (EGFP-F) that remains bound to the plasma membrane in both living and in fixed cells. The EGFP-F coding sequence is preceded by the CMV (cytomegalovirus) promoter that drives high levels of transcription and is followed by the SV40 polyadenylation signal to direct proper processing of the mRNA in eukaryotic cells. The plasmid carries sequences that allow replication in prokaryotic (pUC ori) as well as eukaryotic (SV40 ori) cells and markers that facilitate selection for the plasmid in prokaryotic (kanamycin) cells as well as eukaryotic (neomycin) cells. The presence of EGFP-F can be detected by fluorescence microscopy. (Adapted, with permission, from CLONTECH.)

TABLE 16-4 Some Lipids Used in Lipofection

ABBREVIATION	IUPAC NAME	TYPE	PRODUCT NAME	CELL LINES COMMONLY USED FOR TRANSFECTION
DOTMA	<i>N</i> -[1-(2,3-dioleoyloxy)propyl]- <i>N,N,N</i> -trimethylammonium chloride	monocationic	Lipofectin	AS52 H187 mouse L cells NIH-3T3 HeLa
DOTAP	<i>N</i> -[1-(2,3-dioleoyloxy)propyl]- <i>N,N,N</i> -trimethylammonium methyl sulfate	monocationic	DOTAP	HeLa
DMRIE	1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide	monocationic	DMRIE-C	Jurkat CHO-K1 COS-7 BHK-21
DDAB	dimethyl dioctadecylammonium bromide	monocationic	LipofectACE	COS-7 CHO-K1 BHK-21 mouse L cells
Amidine	<i>N</i> - <i>t</i> -butyl- <i>N'</i> -tetradecyl-3-tetradecyl-aminopropionamide	monocationic	CLONfectin	A-431 HEK293 BHK-21 HeLa L6 CV-1
DC-Cholesterol	3β[<i>N</i> -(<i>N',N'</i> -dimethylaminoethane) carbamoyl]-cholesterol	monocationic	DC-Cholesterol	
DOSPER	1,3-dioleoyloxy-2-(6-carboxyspermyl)propylamide	dicationic	Tfx	CHO HeLa NIH-3T3
DOGS	spermine-5-carboxy-glycine dioctadecyl-amide	polycationic	Transfectam	293 HeLa HepG2 HC11 NIH-3T3
DOSPA	2,3-dioleoyloxy- <i>N</i> -[2(sperminecarbox-amido)ethyl]- <i>N,N</i> -dimethyl-1-propan-aminium trifluoroacetate	polycationic	LipofectAMINE	HT-29 BHK-21 keratinocytes MDCK NIH-3T3
TM-TPS	<i>N,N,N',N''</i> -tetramethyl- <i>N,N',N'',N'''</i> -tetrapalmitylspermine	polycationic	CellFECTIN	CHO-K1 COS-7 BHK-21 Jurkat

METHOD

1. Twenty-four hours before lipofection, harvest exponentially growing mammalian cells by trypsinization and replate them on 60-mm tissue culture dishes at a density of 10^5 cells/dish (or at 5×10^4 cells/35-mm dish). Add 5 ml (or 3 ml for 35-mm dish) of growth medium, and incubate the cultures for 20–24 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.

The cells should be ~75% confluent at the time of lipofection. If the cells are grown for fewer than 12 hours before transfection, they will not be well anchored to the substratum and are likely to detach during exposure to lipid.

2. For each 60-mm dish of cultured cells to be transfected, dilute 1–10 µg of plasmid DNA into 100 µl of sterile deionized H₂O (if using Lipofectin) or 20 mM sodium citrate containing 150 mM NaCl (pH 5.5) (if using Transfectam) in a polystyrene or polypropylene test tube. In a separate tube, dilute 2–50 µl of the lipid solution to a final volume of 100 µl with sterile deionized H₂O or 300 mM NaCl.

▲ IMPORTANT When transfecting with Lipofectin, use polystyrene test tubes; do not use polypropylene tubes, because the cationic lipid DOTMA can bind nonspecifically to polypropylene. For other cationic lipids, use the tubes recommended by the manufacturer.

3. Incubate the tubes for 10 minutes at room temperature.
4. Add the lipid solution to the DNA, and mix the solution by pipetting up and down several times. Incubate the mixture for 10 minutes at room temperature.
5. While the DNA-lipid solution is incubating, wash the cells to be transfected three times with serum-free medium. After the third rinse, add 0.5 ml of serum-free medium to each 60-mm dish and return the washed cells to a 37°C humidified incubator with an atmosphere of 5–7% CO₂.

It is very important to rinse the cells free of serum before the addition of the lipid-DNA liposomes. In some cases, serum is a very effective inhibitor of the transfection process (Felgner and Holm 1989). Similarly, extracellular matrix components such as sulfated proteoglycans can also inhibit lipofection, presumably by binding the DNA-lipid complexes and preventing their interaction with the plasma membranes of the recipient cells.

6. After the DNA-lipid solution has incubated for 10 minutes, add 900 µl of serum-free medium to each tube. Mix the solution by pipetting up and down several times. Incubate the tubes for 10 minutes at room temperature.
7. Transfer each tube of DNA-lipid-medium solution to a 60-mm dish of cells. Incubate the cells for 1–24 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.
8. After the cells have been exposed to the DNA for the appropriate time, wash them three times with serum-free medium. Feed the cells with complete medium and return them to the incubator.
9. If the objective is stable transformation of the cells, proceed to Step 10. Examine the cells 24–96 hours after lipofection using one of the following assays.
 - If a plasmid DNA expressing *E. coli* β-galactosidase was used, follow the steps outlined in Chapter 17, Protocol 7 to measure enzyme activity in cell lysates. Alternatively, carry out a histochemical staining assay as detailed in the panel on **ADDITIONAL PROTOCOL: HISTOCHEMICAL STAINING OF CELL MONOLAYERS FOR β-GALACTOSIDASE**.
 - If a green fluorescence protein expression vector was used, examine the cells with a microscope under 450–490-nm illumination.

- For other gene products, newly synthesized protein may be analyzed by radioimmunoassay, by immunoblotting, by immunoprecipitation following *in vivo* metabolic labeling, or by assays of enzymatic activity in cell extracts.

To minimize dish-to-dish variation in transfection efficiency, it is best to (i) transfect several dishes with each construct, (ii) trypsinize the cells after 24 hours of incubation, (iii) pool the cells, and (iv) replat them on several dishes.

10. To isolate stable transfectants: After the cells have incubated for 48–72 hours in complete medium, trypsinize the cells and replat them in the appropriate selective medium. Change this medium every 2–4 days for 2–3 weeks to remove the debris of dead cells and to allow colonies of resistant cells to grow. Thereafter, individual colonies may be cloned and propagated for assay (for methods, please see Jakoby and Pastan 1979 or Spector et al. 1998b [Chapter 86 in *Cells: A Laboratory Manual*]).

A permanent record of the numbers of colonies may be obtained by fixing the remaining cells with ice-cold methanol for 15 minutes followed by staining with 10% Giemsa for 15 minutes at room temperature before rinsing in tap water. The Giemsa stain should be freshly prepared in phosphate-buffered saline or H₂O and filtered through Whatman No. 1 filter paper before use.

ADDITIONAL PROTOCOL: HISTOCHEMICAL STAINING OF CELL MONOLAYERS FOR β -GALACTOSIDASE

This method, designed for cells growing in 60-mm culture dishes, was adapted from Sanes et al. (1986). Kits that contain all of the necessary reagents for immunohistochemical detection of β -galactosidase are available from several manufacturers.

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <1>.

Additional Materials

Cell fixative

- 2% (v/v) formaldehyde <1>
- 0.2% (v/v) glutaraldehyde <1>
- 1x phosphate-buffered saline

▲ **WARNING** Prepare the cell fixative solution in a chemical fume hood and store at room temperature.

Histochemical stain

- 5 mM potassium ferricyanide (K₃Fe(CN)₆) <1>
- 5 mM potassium ferrocyanide (K₄Fe(CN)₆) <1>
- 2 mM MgCl₂
- 1x phosphate-buffered saline
- 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β -galactoside) <1>

Store the staining solution in the dark at 4°C.

▲ **IMPORTANT** Add the X-gal immediately before applying the stain to the cell monolayer.

Phosphate-buffered saline

Method

1. Wash the transfected cells twice with 2–3 ml of phosphate-buffered saline at room temperature.
2. Add 5 ml of cell fixative to the cells.
3. Wash the cells once with phosphate-buffered saline.
4. Add 3–5 ml of histochemical stain to the cells.
5. Incubate the cells for 14–24 hours at 37°C.
6. Wash the cell monolayer several times with phosphate-buffered saline.
7. Cover the cell monolayer with a small amount of phosphate-buffered saline and examine under a light microscope.

Cells that have expressed the β -galactosidase expression vector should be a brilliant blue. The transfection frequency can be estimated by counting the relative numbers of stained and unstained cells.

Protocol 2

Calcium-phosphate-mediated Transfection of Eukaryotic Cells with Plasmid DNAs

THE UPTAKE OF DNA BY CELLS IN CULTURE IS MARKEDLY ENHANCED when the nucleic acid is presented as a coprecipitate of calcium phosphate and DNA. Graham and van der Eb (1973) initially described this method, and their work laid the foundation for the introduction of cloned DNAs into mammalian cells and led directly to reliable methods for both stable transformation of cells and transient expression of cloned DNAs. For further details on the procedure, please see the information panel on **TRANSFECTION OF MAMMALIAN CELLS WITH CALCIUM PHOSPHATE-DNA COPRECIPITATES** at the end of this chapter.

Since the publication of the original method, increases in the efficiency of the procedure have been achieved by incorporating additional steps, such as a glycerol shock (Parker and Stark 1979) and/or a chloroquine treatment (Luthman and Magnusson 1983) in the transfection protocol. Treatment with sodium butyrate has been shown to enhance the expression of plasmids that contain the SV40 early promoter/enhancer in simian and human cells (Gorman et al. 1983a,b). Transfection kits, which frequently include these and other modifications to the original protocol, are available from a number of companies (please see Table 16-2).

This protocol, which describes a calcium-phosphate-mediated transfection method for use with plasmid DNAs and adherent cells, was modified from Jordan et al. (1996), who rigorously optimized calcium-phosphate-based transfection methods for Chinese hamster ovary cells and human embryonic kidney 293 cells. Following this protocol are variations on this basic method:

- For high-efficiency generation of stable transfectants, please see the alternative method at the end of this protocol.
- For use with high-molecular-weight genomic DNAs (Protocol 3) and for use with adherent cells that have been released from the substratum with trypsin, please see the alternative method at the end of Protocol 3.
- For use with nonadherent cells, please see the alternative method at the end of Protocol 3.

MATERIALS

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

CaCl₂ (2.5 M)

Chloroquine (100 mM) (optional)

Dissolve 52 mg of chloroquine diphosphate in 1 ml of deionized distilled H₂O. Sterilize the solution by passing it through a 0.22- μ m filter; store the filtrate in foil-wrapped tubes at -20°C. Please see Step 5.

Giemsa stain (10% w/v)

The Giemsa stain should be freshly prepared in phosphate-buffered saline or H₂O and filtered through Whatman No. 1 filter paper before use.

Glycerol (15% v/v) in 1x HEPES-buffered saline (optional)

Add 15% (v/v) autoclaved glycerol to filter-sterilized HEPES-buffered saline solution just before use. Please see Step 5.

2x HEPES-buffered saline

140 mM NaCl

1.5 mM Na₂HPO₄·2H₂O

50 mM HEPES

Dissolve 0.8 g of NaCl, 0.027 g of Na₂HPO₄·2H₂O, and 1.2 g of HEPES in a total volume of 90 ml of distilled H₂O. Adjust the pH to 7.05 with 0.5 N NaOH <!>, and then adjust the volume to 100 ml with distilled H₂O. Sterilize the solution by passing it through a 0.22- μ m filter; store the filtrate in 5-ml aliquots at -20°C for periods of up to 1 year.

Methanol <!>

Phosphate-buffered saline

The solution should be sterilized by filtration before use and stored at room temperature.

Sodium butyrate (500 mM) (optional)

In a chemical fume hood, bring an aliquot of stock butyric acid solution to a pH of 7.0 with 10 N NaOH. Sterilize the solution by passing it through a 0.22- μ m filter; store in 1-ml aliquots at -20°C. Please see Step 5.

0.1x TE (pH 7.6)

1 mM Tris-Cl (pH 7.6)

0.1 mM EDTA (pH 7.6)

Sterilize the solution by passing it through a 0.22- μ m filter; store the filtrate in aliquots at 4°C.

Nucleic Acids and Oligonucleotides

Plasmid DNA

Dissolve the DNA in 0.1x TE (pH 7.6) at a concentration of 25 μ g/ml; 50 μ l of plasmid solution is required per milliliter of medium.

To obtain the highest transformation efficiencies, plasmid DNAs should be purified by column chromatography (please see Chapter 1, Protocol 9) or by equilibrium centrifugation in CsCl-ethidium bromide density gradients (please see Chapter 1, Protocol 10). If the starting amount of plasmid DNA is limiting, then add carrier DNA to adjust the final concentration to 25 μ g/ml. Eukaryotic carrier DNA prepared in the laboratory usually gives higher transfection efficiencies than commercially available DNA such as calf thymus or salmon sperm DNA. Sterilize the carrier DNA before use by ethanol precipitation or extraction with chloroform.

Media

Cell culture growth medium (complete and [optional] selective)

Special Equipment

Tissue culture dishes (60-mm) or 12-well plates

This protocol is designed for cells grown in 60-mm culture dishes or 12-well plates. If other multiwell plates, flasks, or dishes of a different diameter are used, scale the cell density and reagent volumes accordingly. Please see Table 16-3.

Additional Reagents

Step 6 of this protocol requires the reagents listed in Chapter 6, Protocol 10, and Chapter 7, Protocol 8.

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. Twenty-four hours before transfection, harvest exponentially growing cells by trypsinization and replate them at a density of 1×10^5 to 4×10^5 cells/cm² in 60-mm tissue culture dishes or 12-well plates in the appropriate complete medium. Incubate the cultures for 20–24 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂. Change the medium 1 hour before transfection.

To obtain optimum transfection frequencies, it is important to use exponentially growing cells. Cell lines used for transfection should never be allowed to grow to >80% confluency.

2. Prepare the calcium phosphate–DNA coprecipitate as follows: Combine 100 µl of 2.5 M CaCl₂ with 25 µg of plasmid DNA in a sterile 5-ml plastic tube and, if necessary, bring the final volume to 1 ml with 0.1× TE (pH 7.6). Mix 1 volume of this 2× calcium–DNA solution with an equal volume of 2× HEPES-buffered saline at room temperature. Quickly tap the side of the tube to mix the ingredients and allow the solution to stand for 1 minute.

The precipitation reaction mixture can be doubled or quadrupled in volume if a larger number of cells are to be transfected. Normally, 0.1 ml of the calcium phosphate–DNA coprecipitate is added per 1 ml of medium in the culture dish, well, or flask.

3. Immediately transfer the calcium phosphate–DNA suspension into the medium above the cell monolayer. Use 0.1 ml of suspension for each 1 ml of medium in a well or 60-mm dish. Rock the plate gently to mix the medium, which will become yellow-orange and turbid. Carry out this step as quickly as possible because the efficiency of transfection declines rapidly once the DNA precipitate is formed. If the cells will be treated with chloroquine, glycerol, and/or sodium butyrate, proceed directly to Step 5.

In some instances, higher transfection frequencies are achieved by first removing the medium and then directly adding the calcium phosphate–DNA suspension to the exposed cells. Thereafter, incubate the cells for 15 minutes at room temperature, and then add medium to the dish.

4. Transfected cells that will not be treated with transfection facilitators should be incubated at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂. After 2–6 hours incubation, remove the medium and DNA precipitate by aspiration. Add 5 ml of warmed (37°C) complete growth medium and return the cells to the incubator for 1–6 days. Proceed to Step 6 to assay for transient expression of the transfected DNA, or proceed directly to Step 7 if the objective is stable transformation of the cells.

5. The uptake of DNA can be increased by treatment of the cells with chloroquine in the presence of the calcium phosphate-DNA coprecipitate or exposure to glycerol and sodium butyrate following removal of the coprecipitate solution from the medium.

TREATMENT OF CELLS WITH CHLOROQUINE

Chloroquine is a weak base that is postulated to act by inhibiting the intracellular degradation of the DNA by lysosomal hydrolases (Luthman and Magnusson 1983). The concentration of chloroquine added to the growth medium and the time of treatment are limited by the sensitivity of the cells to the toxic effect of the drug. The optimal concentration of chloroquine for the cell type used should be determined empirically (please see the information panel on **CHLOROQUINE DIPHOSPHATE**).

- a. Dilute 100 mM chloroquine diphosphate 1:1000 directly into the medium either before or after the addition of the calcium phosphate-DNA coprecipitate to the cells.
- b. Incubate the cells for 3–5 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.

Most cells can survive in the presence of chloroquine for 3–5 hours. Cells often develop a vesicularized appearance during treatment with chloroquine.

- c. After the treatment with DNA and chloroquine, remove the medium, wash the cells with phosphate-buffered saline, and add 5 ml of warmed complete growth medium. Return the cells to the incubator for 1–6 days. Proceed to Step 6 to assay for transient expression of the transfected DNA, or proceed directly to Step 7 if the objective is stable transformation of the cells.

TREATMENT OF CELLS WITH GLYCEROL

This procedure may be used following treatment with chloroquine. Because cells vary widely in their sensitivity to the toxic effects of glycerol, each cell type must be tested in advance to determine the optimum time (30 seconds to 3 minutes) of treatment.

- a. After cells have been exposed for 2–6 hours to the calcium phosphate-DNA coprecipitate in growth medium (\pm chloroquine), remove the medium by aspiration and wash the monolayer once with phosphate-buffered saline.
- b. Add 1.5 ml of 15% glycerol in 1x HEPES-buffered saline to each monolayer, and incubate the cells for the predetermined optimum length of time at 37°C.
- c. Remove the glycerol by aspiration, and wash the monolayers once with phosphate-buffered saline.
- d. Add 5 ml of warmed complete growth medium, and incubate the cells for 1–6 days. Proceed to Step 6 to assay for transient expression of the transfected DNA, or proceed directly to Step 7 if the objective is stable transformation of the cells.

TREATMENT OF CELLS WITH SODIUM BUTYRATE

The mechanism through which sodium butyrate acts is not known with certainty; however, the compound is an inhibitor of histone deacetylation (Lea and Randolph 1998), which suggests that treatment may lead to histone hyperacetylation and a chromatin structure on the incoming plasmid DNA that is predisposed to transcription (Workman and Kingston 1998).

- a. Following the glycerol shock, dilute 500 mM sodium butyrate directly into the growth medium (Step d, treatment of cells with glycerol). Different concentrations of sodium butyrate are used depending on the cell type. For example:

CV-1	10 mM
NIH-3T3	7 mM
HeLa	5 mM
CHO	2 mM

The correct amount for other cell lines that may be transfected should be determined empirically.

- b. Incubate the cells for 1–6 days. Proceed to Step 6 to assay for transient expression of the transfected DNA, or proceed directly to Step 7 if the objective is stable transformation of the cells.
6. To assay the transfected cells for transient expression of the introduced DNA, harvest the cells 1–6 days after transfection. Analyze RNA or DNA using hybridization. Analyze newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following *in vivo* metabolic labeling, or by assays of enzymatic activity in cell extracts.
To minimize dish-to-dish variation in transfection efficiency, it is best to (i) transfect several dishes with each construct, (ii) trypsinize the cells after 24 hours of incubation, (iii) pool the cells, and (iv) replat them on several dishes.
7. To isolate stable transfectants:
 - a. Incubate the cells for 24–48 hours in nonselective medium to allow time for expression of the transferred gene(s).
 - b. Either trypsinize and replat the cells in the appropriate selective medium or add the selective medium directly to the cells without further manipulation.
 - c. Change the selective medium with care every 2–4 days for 2–3 weeks to remove the debris of dead cells and to allow colonies of resistant cells to grow.
 - d. Clone individual colonies and propagate for assay (for methods, please see Jakoby and Pastan 1979 or Spector et al. 1998b [Chapter 86 of *Cells: A Laboratory Manual*]).
 - e. Obtain a permanent record of the numbers of colonies by fixing the remaining cells with ice-cold methanol for 15 minutes followed by staining with 10% Giemsa for 15 minutes at room temperature before rinsing in tap water.

The dilution at which the transfected cells should be replated to yield well-separated colonies is determined by the efficiency of stable transformation, which can vary over several orders of magnitude (e.g., please see Spandidos and Wilkie 1984). The efficiency is dependent on the recipient cell type (significant differences have been observed even between different clones or different passage numbers of the same cell line [Corsaro and Pearson 1981; Van Pel et al. 1985]), the nature of the introduced gene and the efficacy of the transcriptional control signals associated with it, and the amount of DNA used in the transfection.

ALTERNATIVE PROTOCOL: HIGH-EFFICIENCY CALCIUM-PHOSPHATE-MEDIATED TRANSFECTION OF EUKARYOTIC CELLS WITH PLASMID DNAs

A modification of the classical calcium phosphate transfection method that greatly enhances the efficiency of the procedure was developed by Hiroto Okayama and colleagues (Chen and Okayama 1987, 1988). Their method works particularly well when stable transfectants are to be isolated using supercoiled plasmid DNAs and differs from the classical procedure in that the calcium phosphate-DNA coprecipitate is allowed to form in the tissue culture medium during prolonged incubation (15–24 hours) under controlled conditions of pH (6.96) and reduced CO₂ tension (2–4%).

Variables Affecting the Efficiency of Transfection

Variables that affect transfection include the purity, form, and amount of the DNA; the pH of the 2x BES buffer; and the concentration of CO₂ in the incubator.

- Impure plasmid DNAs transfect poorly because of the inhibitory effects of bacterial contaminants. For this reason, the best results are obtained with scrupulously clean DNA, preferably purified through specialized chromatography resins or two rounds of CsCl centrifugation (please see Chapter 1, Protocols 9 and 10). If necessary, the plasmid can be further purified by phenol:chloroform extraction in the presence of 1% (w/v) SDS.
- Linear DNAs yield very low transformation frequencies, perhaps because the slow formation of the calcium phosphate-DNA coprecipitate leaves the DNA exposed for long periods of time to cell nucleases.
- The nature of the precipitate is affected by the amount of DNA used. A transition (visible under the microscope) from a coarse precipitate to a fine precipitate occurs at the optimal DNA concentration (usually 2–3 µg/ml in the growth medium). The optimum DNA concentration encompasses a narrow range and should be determined empirically for individual cell lines.
- The slow formation of the calcium phosphate-DNA coprecipitate requires a slightly acidic pH and incubation in an atmosphere containing low concentrations of CO₂. The pH curve is very sharp with a clearly defined optimum at 6.96, whereas the CO₂ concentration is optimal between 2% and 4%.

Chen and Okayama (1987) reported that this method could be used for transient analysis of gene expression and that the simultaneous introduction of two or more plasmids reduced the overall efficiency of transfection. The overall frequency was still much higher than that obtained with other calcium phosphate methods. When cotransfecting with a selectable marker, it is usually necessary to optimize the system using mixtures containing different ratios of plasmids carrying the selectable marker of the gene of interest (e.g., 1:2, 1:5, and 1:10).

Additional Materials**2x BES-buffered saline (BBS)**

50 mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid)

280 mM NaCl

1.5 mM Na₂HPO₄·2H₂O

Dissolve 1.07 g of BES, 1.6 g of NaCl, and 0.027 g of Na₂HPO₄ in a total volume of 90 ml of distilled H₂O. Adjust the pH of the solution to 6.96 with HCl at room temperature, and then adjust the volume to 100 ml with distilled H₂O. Sterilize the solution by passing it through a 0.22-µm filter; store the filtrate in aliquots at -20°C.

CaCl₂ (0.25 M)

Dissolve 1.1 g of CaCl₂·6H₂O in 20 ml of distilled H₂O. Sterilize the solution by passing it through a 0.22-µm filter. Store the filtrate in 1-ml aliquots at -20°C.

Superhelical plasmid at 1 µg/µl in 0.1x TE (pH 7.6)**Tissue culture dishes (90-mm)**

This protocol is designed for cells grown in 90-mm culture dishes. If other multiwell plates, flasks, or dishes of a different diameter are used, scale the cell density and reagent volumes accordingly. Please see Table 16-3.

Method

1. Twenty-four hours before transfection, harvest exponentially growing cells by trypsinization and replate aliquots of 5×10^5 cells onto 90-mm tissue culture dishes. Add 10 ml of complete growth medium, and incubate the cultures overnight at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.

2. Mix 20–30 µg of superhelical plasmid DNA with 0.5 ml of 0.25 M CaCl₂. Add 0.5 ml of 2x BES-buffered saline (BBS) and incubate the mixture for 10–20 minutes at room temperature. Do not expect a visible precipitate to form during this time.
3. Add the CaCl₂-DNA-BBS solution dropwise to the dishes of cells, swirling gently to mix well. Incubate the cultures for 15–24 hours at 37°C in a humidified incubator in an atmosphere of 2–4% CO₂.
4. Remove the medium by aspiration, and rinse the cells twice with medium. Add 10 ml of nonselective medium, and incubate the cultures for 18–24 hours at 37°C in a humidified incubator in an atmosphere of 5% CO₂.
5. Following 18–24 hours of incubation in nonselective medium, to allow expression of the transfected gene(s) to occur, trypsinize and replat the cells in the appropriate selective medium. Change the selective medium with care every 2–4 days for 2–3 weeks to remove the debris of dead cells and to allow colonies of resistant cells to grow.

The dilution at which the transfected cells should be replated to yield well-separated colonies is determined by the efficiency of stable transformation, which can vary over several orders of magnitude (e.g., please see Spandidos and Wilkie 1984). The efficiency is dependent on: the recipient cell type (significant differences have been observed even between different clones or different passage numbers of the same cell line [Corsaro and Pearson 1981; Van Pel et al. 1985]), the nature of the introduced gene and the efficacy of the transcriptional control signals associated with it, and the amount of donor DNA used in the transfection.

6. Thereafter, clone individual colonies and propagate for assay (for methods, please see Jakoby and Pastan 1979 or Spector et al. 1998b [Chapter 86 in *Cells: A Laboratory Manual*]).

A permanent record of the number of colonies may be obtained by fixing the remaining cells with ice-cold methanol for 15 minutes followed by staining with 10% Giemsa for 15 minutes at room temperature before rinsing in tap water. The Giemsa stain should be freshly prepared in phosphate-buffered saline or H₂O and filtered through Whatman No. 1 filter paper before use.

Protocol 3

Calcium-phosphate-mediated Transfection of Cells with High-molecular-weight Genomic DNA

MAMMALIAN GENES HAVE BEEN SUCCESSFULLY ISOLATED by transfecting cultured mammalian cells with genomic DNA, followed by selection for the gene of interest. This includes dominant cellular oncogenes, genes that encode cell surface molecules, and, as selection/identification strategies and techniques have improved, genes that encode intracellular proteins. Target genes are recovered from the chromosomal DNA of stably transfected cells by virtue of their species-specific repetitive DNA elements or by linkage to cotransfected plasmid DNAs.

The method outlined below is a modification of the calcium phosphate procedure described by Graham and Van der Eb (1973), using high-molecular-weight genomic DNA instead of plasmid DNA. The procedure works especially well to generate stable lines of cells carrying transfected genes that complement mutations in the hosts' chromosomal genes (Sege et al. 1984; Kingsley et al. 1986). This protocol was supplied by P. Reddy and M. Krieger (Massachusetts Institute of Technology).

MATERIALS

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

CaCl₂ (2 M)

Sterilize by filtration, and store frozen as 5-ml aliquots.

Glycerol (15% v/v) in 1× HEPES-buffered saline

Add 15% (v/v) autoclaved glycerol to filter-sterilized HEPES-buffered saline solution just before use.

HEPES-buffered saline

21 mM HEPES
0.7 mM Na_2HPO_4
137 mM NaCl
5 mM KCl
6 mM dextrose

Adjust the pH of the solution to 7.10. Sterilize the solution by filtration and store frozen in 25–50-ml aliquots. Thaw a fresh aliquot before each transfection and check the pH of a small volume. Readjust pH if necessary to 7.10.

Isopropanol

NaCl (3 M)

Sterilize by filtration, and store at room temperature.

Nucleic Acids and Oligonucleotides

Genomic DNA

Prepare high-molecular-weight DNA in TE from appropriate cells as described in Chapter 6, Protocol 3. Dilute the DNA to 100 $\mu\text{g}/\text{ml}$ in TE (pH 7.6). Approximately 20–25 μg of genomic DNA is required to transfect each 90-mm plate of cultured cells.

The genomic DNA must be sheared to a size range of 45–60 kb before using it to transfect cells (please see Steps 2 and 3). The appropriate conditions for shearing the genomic DNA are best determined in preliminary experiments as follows: Shear 2-ml aliquots of high-molecular-weight DNA by passing each aliquot through a 22-gauge needle for a different number of times (e.g., three, four, five, or six times). Examine the DNA by electrophoresis on a 0.6% (w/v) agarose gel followed by staining with either ethidium bromide or SYBR Gold. As markers, use monomeric and dimeric forms of linear bacteriophage λ DNA. To optimize the remaining steps, sheared DNA of the proper size should then be taken through Step 9 of the protocol using dishes without cells.

Plasmid with selectable marker

Optional, please see notes to Steps 3 and 12.

Media

Cell culture growth medium (complete and selective)

Special Equipment

Polyethylene tubes (12-ml)

Shepherd's crook

Siliconized glass Pasteur pipette containing a hook at the end.

Tissue culture dishes (90-mm)

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. On day 1 of the experiment, plate exponentially growing cells (e.g., CHO cells) at a density of 5×10^5 cells per 90-mm culture dish in appropriate growth medium containing serum. Incubate the cultures for ~16 hours at 37°C in a humidified incubator with an atmosphere of 5% CO_2 .
2. On day 2, shear an appropriate amount of high-molecular-weight DNA into fragments ranging in size from 45 kb to 60 kb, by passing it through a 22-gauge needle for the predetermined number of times (please see the note to the Genomic DNA entry above in Materials).
Cells should be transfected with 20–25 μg of genomic DNA per 90-mm dish.

3. Precipitate the sheared DNA by adding 0.1 volume of 3 M NaCl and 1 volume of isopropanol. Collect the DNA on a Shepherd's crook. Drain the precipitate briefly against the side of the tube and transfer it to a second tube containing HEPES-buffered saline (1 ml per 12–15 µg of DNA). Redissolve the DNA by gentle rotation for 2 hours at 37°C. Make sure that all of the DNA has dissolved before proceeding.

When cotransfecting with a selectable marker (please see the note below Step 12), add to the genomic DNA a sterile solution of the appropriate plasmid to a final concentration of 0.5 µg/ml.

4. Transfer 3-ml aliquots of sheared genomic DNA into 12-ml polyethylene tubes (one aliquot per two dishes to be transfected).

The number of dishes required and transfectants obtained will vary from one cell line to another and on the efficiency of the selection method. As a guide, ~15–20 dishes of CHO cells must be transfected to obtain 3–10 stable transformants.

5. To form the calcium phosphate–DNA coprecipitate, gently vortex an aliquot of sheared genomic DNA, and add 120 µl of 2 M CaCl₂ in a dropwise fashion. Incubate the tube for 15–20 minutes at room temperature.

The solution should turn hazy, but it should not form visible clumps of precipitate.

6. Aspirate the medium from two dishes of cells (from Step 1) and gently add 1.5 ml of the calcium phosphate–DNA coprecipitate to each dish. Carefully rotate the dishes to swirl the medium and spread the precipitate over the monolayer of cells. Incubate the cells for 20 minutes at room temperature, rotating the dishes once during the incubation.

7. Gently add 10 ml of warmed (37°C) growth medium to each dish and incubate for 6 hours at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

8. Repeat Steps 5–7 until all of the dishes of cells contain the calcium phosphate–DNA precipitate.

9. After 6 hours of incubation, examine each dish under a light microscope. A “peppery” precipitate should be seen adhering to the cells. The precipitate should be neither too fine nor clumpy.

Experience will dictate how a “peppery” precipitate looks under the microscope. Terminate the experiment with cells if a very fine or clumpy precipitate is visualized at this step. The failure to form a peppery precipitate at this step or a hazy solution at Step 5 could be due to the use of a HEPES-buffered saline solution of improper pH, an overly long incubation at Step 5, or a suboptimal concentration of CaCl₂ or DNA.

10. In most cases, treatment with glycerol at this step will enhance the transfection frequency. To shock the cells with glycerol:

- a. Aspirate the medium containing the calcium phosphate–DNA coprecipitate.
- b. To each dish of cells, add 3 ml of 15% glycerol in 1x HEPES-buffered saline that has been warmed to 37°C. Incubate for *no longer than 3 minutes* at room temperature.

It is important that the glycerol in the HEPES-buffered saline *not* be left in contact with the cells for too long. The optimum time period usually spans a narrow range and varies from one cell line to another and from one laboratory to the next. For these reasons, treat only a few dishes at a time and take into account the length of time to aspirate the glycerol in the HEPES-buffered saline. Do not to exceed the optimum incubation period. Seconds can count!

- c. Aspirate the glycerol in the HEPES-buffered saline and rapidly wash the dishes twice with 10 ml of warmed growth medium.
- d. Add 10 ml warmed growth medium and incubate the cultures for 12–15 hours at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

11. Replace the medium with 10 ml of fresh growth medium. Continue the incubation overnight at 37°C in a humidified incubator with an atmosphere of 5% CO₂.
12. Microscopic examination of cells at this point (day 4) should reveal a normal morphology. Cells can be trypsinized and replated in selective medium on day 4. Continue the incubation for 2–3 weeks to allow growth of complemented and/or resistant colonies. Change the medium every 2–3 days.

The length of the selection period, the cell density of replating, and the selection conditions all depend on the mutation or gene being complemented or selected. Optimum cell density for replating at Step 12 usually varies between 2.5×10^5 and 1×10^6 cells per 90-mm dish. Determine this parameter empirically by plating different numbers of cells without transfection and applying the selection procedure. For logistical reasons, use the highest density that still allows efficient cell killing.

Cotransfection (e.g., with a plasmid conferring G418 resistance) can be used to distinguish between transfectants and revertants. Because the reversion frequency for some mutant cell lines can be as high as 10^{-6} (i.e., 1 per 1 million cells plated), false positives can be a problem. The transfection frequency is usually 2×10^{-7} , and the cotransfection frequency is $\sim 10^{-8}$. The use of a selection (e.g., G418 resistance) in conjunction with the mutation/gene selection should eliminate false positives. For further details, please see the information panel on **COTRANSFORMATION**.

13. Thereafter, clone individual colonies and propagate them for assay (for methods, please see Jakoby and Pastan 1979 and Step 7d, p. 16.18).

A permanent record of the numbers of colonies may be obtained by fixing the remaining cells with ice-cold methanol for 15 minutes followed by staining with 10% Giemsa for 15 minutes at room temperature before rinsing in tap water. The Giemsa stain should be freshly prepared in PBS or H₂O and filtered through Whatman No. 1 filter paper before use.

ALTERNATIVE PROTOCOL: CALCIUM-PHOSPHATE-MEDIATED TRANSFECTION OF ADHERENT CELLS

This protocol can be used with all types of adherent cells, but is particularly useful for polarized epithelial cells, which do not efficiently take up material by endocytosis through the apical plasma membrane.

Additional Materials

Exponentially growing adherent mammalian cells
Sorvall H1000B rotor or equivalent

Method

1. Harvest exponentially growing adherent cells by trypsinization. Resuspend the cells in growth medium containing serum, and centrifuge aliquots containing $\sim 10^6$ cells at 800g (2000 rpm in a Sorvall H1000B rotor) for 5 minutes at 4°C. Discard the supernatants.

2. Form the calcium phosphate-DNA coprecipitate as described in Protocol 2, Step 2 if plasmid DNA is used or as described in Protocol 3, Step 5 if genomic DNA is used.

Note that the coprecipitate with plasmid DNA takes only ~ 5 minutes to prepare, whereas the coprecipitate containing genomic DNA takes ~ 25 minutes to prepare. Execute the initial two steps of this protocol so that cells and coprecipitate are ready at the same time.

3. Resuspend each aliquot of 10^6 cells in 0.5 ml of the calcium phosphate-DNA suspension, and incubate for 15 minutes at room temperature.

This protocol can be easily modified to accommodate greater numbers of cells. For example, Chu and Sharp (1981) used 10^6 cells in 2 ml of calcium phosphate-DNA suspension containing 25 μ g of DNA. In this case, after 15 minutes, dilute the mixture with 40 ml of complete growth medium supplemented with 0.05x HEPES-buffered saline and 6.25 mM CaCl_2 . Plate the cells at a density of 5×10^4 cells per 150-mm dish.

4. To each aliquot, add 4.5 ml of warmed growth medium (with or without chloroquine; please see Protocol 2, Step 5), and plate the entire suspension (~ 5 ml) in a single 90-mm tissue culture dish. Incubate the cells for up to 24 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO_2 .

5. Some types of cells may be further treated with glycerol and sodium butyrate to facilitate transfection. Please follow the procedures in Protocol 2, Step 5.

6. Thereafter, assay the cells for transient expression or place in the appropriate selective medium for the isolation of stable transformants (please see Protocol 2, Steps 6 and 7).

ALTERNATIVE PROTOCOL: CALCIUM-PHOSPHATE-MEDIATED TRANSFECTION OF CELLS GROWING IN SUSPENSION

A few cell lines grown as suspension cultures (e.g., HeLa cells) can be transfected using the modified calcium phosphate procedure described in this protocol. However, most lines of cells grown in suspension are resistant to calcium-phosphate-mediated transfection methods. Intransigent cell lines are best transfected using electroporation (Protocol 5) or lipofection (Protocol 1).

Additional Materials

Exponentially growing mammalian suspension cells

Phosphate-buffered saline (PBS)

Sorvall H1000B rotor or equivalent

Method

1. Collect cells from an exponentially growing suspension culture by centrifugation at 800g (2000 rpm in a Sorvall H1000B rotor) for 5 minutes at 4°C. Discard the supernatant, and resuspend the cell pellet in 20 volumes of ice-cold PBS. Divide the suspension into aliquots containing 1×10^6 cells each. Recover the washed cells by centrifugation as before, and again discard the supernatant.
2. Form the calcium phosphate-DNA coprecipitate as described in Protocol 2, Step 2 if plasmid DNA is used for transfection or as described in Protocol 3, Step 5 if genomic DNA is used.
 Note that preparation of the coprecipitate with plasmid DNA takes only ~5 minutes to prepare, whereas the coprecipitate containing genomic DNA takes ~25 minutes to prepare. Execute the initial two steps of this protocol so that cells and coprecipitate are ready at the same time.
3. Gently resuspend 1×10^6 cells in 1 ml of calcium phosphate-DNA suspension (containing ~20 µg of DNA), and allow the suspension to stand for 20 minutes at room temperature.
4. Add 10 ml of complete growth medium (with or without chloroquine; please see Protocol 2, Step 5) to a tube of cells, and plate the entire suspension in a single 90-mm tissue culture dish. Incubate the cells for 6–24 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.
5. (Optional for cells known to survive a glycerol shock) At 4–6 hours after beginning Step 4, carry out the following (otherwise, proceed to Step 6):
 - a. Collect the cells by centrifugation at 800g (2000 rpm in a Sorvall H1000B rotor) for 5 minutes at room temperature, and wash them once with PBS.
 - b. Resuspend the washed cells in 1 ml of 15% glycerol in 1x HEPES-buffered saline, and incubate the cells for 30 seconds to 3 minutes at 37°C.
 Please see the note to Step 10b in the main protocol.
 - c. Dilute the suspension with 10 ml of PBS, and recover the cells by centrifugation as described in Step a. Wash the cells once in PBS.
 - d. Resuspend the washed cells in 10 ml of complete growth medium, and plate them in a 90-mm tissue culture dish. Incubate the culture for 48 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.
6. Recover the cells by centrifugation at 800g (2000 rpm in a Sorvall H1000B rotor) for 5 minutes at room temperature, and wash them once with PBS.
7. Resuspend the cells in 10 ml of complete growth medium warmed to 37°C. Return the cells to the incubator for 48 hours before assaying for transient expression of transfected genes (Protocol 2, Step 6) or replating the cells in selective medium for isolation of stable transformants (Protocol 2, Step 7).

Protocol 4

Transfection Mediated by DEAE-Dextran: High-efficiency Method

THE FIRST TRANSFECTION METHODS, DEVELOPED IN THE LATE 1950s, used hyperosmotic and polycationic proteins to promote entry of DNA into cells (for review, please see Felgner 1990). The results were erratic, and the efficiency of transfection was, at best, very poor. The situation improved dramatically in the mid 1960s when DEAE-dextran (diethylaminoethyl-dextran) was used to introduce poliovirus RNA (Pagano and Vaheiri 1965) and SV40 and polyomavirus DNAs (McCutchan and Pagano 1968; Warden and Thorne 1968) into cells. The procedure, with slight modifications, continues to be widely used for transfection of cultured cells with viral genomes and recombinant plasmids. Although the mechanism of action of DEAE-dextran is not understood in detail, it seems likely that the high-molecular-weight positively charged polymer serves as a bridge between the negatively charged nucleic acid and the negatively charged surface of the cell (Lieber et al. 1987; Holter et al. 1989). After the DEAE-dextran/DNA complexes have been internalized by endocytosis (Ryser 1967; Yang and Yang 1997), the DNA somehow escapes from the increasingly acidic endosomes and is transported by unknown mechanisms across the cytoplasm and into the nucleus.

Transfection mediated by DEAE-dextran differs from calcium phosphate coprecipitation in three important respects. First, it is used for transient expression of cloned genes and not for stable transformation of cells (Gluzman 1981). Second, it works very efficiently with lines of cells such as BSC-1, CV-1, and COS but is unsatisfactory with many other types of cells. Third, smaller amounts of DNA are used for transfection with DEAE-dextran than with calcium phosphate coprecipitation. Maximal transfection efficiency of 10^5 simian cells is achieved with 0.1–1.0 μ g of supercoiled plasmid DNA; larger amounts of DNA (>2–3 μ g) can be inhibitory. By contrast to transfection mediated by calcium phosphate, where high concentrations of DNA are required to promote the formation of a coprecipitate, carrier DNA is rarely used with the DEAE-dextran transfection method.

Since the method was introduced more than 20 years ago, many variants of DEAE-dextran transfection have been described. In most cases, the cells are exposed to a preformed mixture of DNA and high-molecular-weight DEAE-dextran (m.w. >500,000). However, a modified procedure has been described in which the cells are exposed first to DEAE-dextran and then to DNA (al-Moslih and Dubes 1973; Holter et al. 1989). All of these methods seek to maximize the uptake of DNA and to minimize the cytotoxic effects of DEAE-dextran. The following are among the variables that influence the efficiency of transfection.

- **Concentration of DEAE-dextran used and length of time cells are exposed to it.** It is possible to use either a relatively high concentration of DEAE-dextran (1 mg/ml) for short periods (30 minutes to 1.5 hours) or a lower concentration (250 μ g/ml) for longer periods of time (up to

8 hours). The first of these transfection procedures is the more efficient, but it involves monitoring the cells for early signs of distress when they are exposed to the DNA/DEAE-dextran mixture. The second technique is less demanding and more reliable, but slightly less efficient. However, it can be combined with shock treatments (see below) that can raise the efficiency of transfection to very high levels.

- **Use of facilitators such as DMSO, chloroquine, or glycerol.** The efficiency of transient expression of genes introduced by DEAE transfection is increased ~50-fold if cells are exposed to DMSO, glycerol, polyethyleneimine, or other substances such as Starburst dendrimers that perturb osmosis and increase the efficiency of endocytosis (Lopata et al. 1984; Sussman and Milman 1984; Kukowska-Latello et al. 1996; Zauner et al. 1996; Godbey et al. 1999). A similar increase in efficiency of transfection of some lines of cultured cells may be obtained by exposing the transfected cells to chloroquine, which prevents acidification of endosomes and promotes early release of DNA into the cytoplasm (Luthman and Magnusson 1983). In the best cases, 80% of the cells in a transfected population can express foreign genes when DEAE-dextran and facilitators are used in combination (e.g., please see Kluxen and Lübbert 1993). However, the efficiency of DNA transfection using DEAE-dextran with a facilitator varies greatly from cell line to cell line. Conditions that are optimal for one cell line may not work at all for another. To obtain consistently high efficiencies of transformation with a particular cell line, the following factors should be standardized:

Density of cells and their state of growth.

Amount of transfecting DNA.

Concentration and molecular weight of DEAE-dextran.

Length of time cells are exposed to DNA.

Whether the DEAE-dextran and DNA are added to the cells simultaneously or sequentially (al-Moslih and Dubes 1973; Holter et al. 1989).

Length and temperature of the posttransfection facilitation and the concentration of the facilitating agent.

Whether the cells are transfected while growing on a solid support or are first removed from the solid support and transfected in suspension (Golub et al. 1989).

For publications that analyze the effects of some or all of these conditions on transfection efficiency, please see Holter et al. (1989), Fregeau and Bleackley (1991), Kluxen and Lübbert (1993), and Luo and Saltzman (1999).

In addition to its use as a primary agent for transfection, DEAE-dextran can also be used as an adjuvant to enhance the efficiency of electroporation. Although the effects appear to vary from one cell line to another, the combination of electroporation and DEAE-dextran in some cases can improve the efficiency of transfection by a factor of 10–100 (Gauss and Leiber 1992).

DNA transfected into cells by the DEAE-dextran method is prone to mutation. This is particularly true of sequences cloned in vectors that can replicate in transfected mammalian cells. For example, when the *E. coli lacI* gene, cloned in a plasmid containing an SV40 origin of replication, was introduced into COS-7 cells, allowed to replicate for several generations, and then returned to *E. coli*, mutations occurred at a frequency of one to several percent (Calos et al. 1983). Stunning in their variety, mutations induced during replication in mammalian cells include deletions, insertions, and base substitutions (Razzaque et al. 1983; Lebkowski et al. 1984; Ashman and Davidson 1985). These mutations are thought to arise as a consequence of damage caused by the action of degradative enzymes and low pH in the lysosomes and also perhaps by the lack of a complete chromatin structure after the transfecting DNA enters the nucleus (Miller et al. 1984; Reeves et al. 1985).

Here, we describe two variations on the classical DEAE-dextran transfection procedure. The first (main protocol) involves a brief exposure of cells to a high concentration of DEAE-dextran and yields higher transfection frequencies but elevated cellular toxicity. The second (please see the panel on **ALTERNATIVE PROTOCOL: TRANSFECTION MEDIATED BY DEAE-DEXTRAN: INCREASED CELL VIABILITY** at the end of this protocol) involves a longer exposure of cells to a lower concentration of DEAE-dextran, which produces lower transfection frequencies but increased cell survival.

TRANSFECTION OF COS CELLS

The DEAE-dextran procedure is most often used to transfect simian COS cells. These cells were developed by Gluzman (1981) and express the SV40 large T antigen (please see the information panel on **COS CELLS** in Chapter 11). Introduction of the SV40 origin of replication, typically by use of the SV40 early region promoter-enhancer/origin to express the gene or cDNA of interest, results in the amplification of the origin-containing plasmid to very high copy number (Gluzman 1981). This amplification in turn produces a high level of expression of the transfected cDNA or gene, but severely stresses and eventually kills cells that take up the plasmid. COS cells are thus usually used as transient transfection hosts and analyzed 48–72 hours posttransfection.

The efficiency of DEAE-dextran-mediated transfection of COS cells is very high, often approaching 50% of the cells on a dish. For this reason, COS cells are frequently used in expression cloning (please see Chapter 11, Protocol 2). The high efficiency of transfection also allows multiple plasmids to be introduced simultaneously into the cells. For example, entire intermediary metabolism pathways can be reconstituted in COS cells by introducing expression plasmids encoding individual enzymes in the pathway (Zuber et al. 1988).

MATERIALS

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

Chloroquine diphosphate (100 mM)

Dissolve 60 mg of chloroquine diphosphate in 1 ml of deionized distilled H₂O. Sterilize the solution by passing it through a 0.22- μ m filter. Store the filtrate in foil-wrapped tubes at –20°C.

Please see the information panel on **CHLOROQUINE DISPHOSPHATE**.

DEAE-dextran (50 mg/ml)

Dissolve 100 mg of DEAE-dextran ($M_v = 500,000$; Pharmacia) in 2 ml of distilled H₂O. Sterilize the solution by autoclaving for 20 minutes at 15 psi (1.05 kg/cm²) on liquid cycle. Autoclaving also assists dissolution of the polymer.

The molecular weight of the DEAE-dextran originally used for transfection was $>2 \times 10^6$ (McCutchan and Pagano 1968). Although this material is no longer available commercially, it is still occasionally found in chemical storerooms. The older batches of higher-molecular-weight DEAE-dextran are more efficient for transfection than the lower-molecular-weight polymers currently available.

Phosphate-buffered saline (PBS)

Sterilize the solution by filtration before use and store it at room temperature.

Tris-buffered saline with dextrose (TBS-D)

Immediately before use, add 20% (w/v) dextrose (prepared in H₂O and sterilized by autoclaving or filtration) to the TBS solution. The final dextrose concentration should be 0.1% (v/v).

Nucleic Acids and Oligonucleotides

Plasmid DNA

To obtain the highest transformation efficiencies, purify the plasmid DNAs by column chromatography (please see Chapter 1, Protocol 9) or by equilibrium centrifugation in CsCl–ethidium bromide density gradients (please see Chapter 1, Protocol 10).

Media

Cell culture growth medium (complete and serum-free)

Special Equipment

Tissue culture dishes (60-mm or 35-mm)

This protocol is designed for cells grown in 60-mm or 35-mm culture dishes. If multiwell plates, flasks, or dishes of a different diameter are used, scale the cell density and reagent volumes accordingly. Please see Table 16-3.

Additional Reagents

Step 8 of this protocol requires the reagents listed in Chapter 6, Protocol 1, and Chapter 7, Protocol 8.

Cells and Tissues

Exponentially growing cultures of mammalian cells

DEAE-DEXTRAN TRANSFECTION KITS

Several manufacturers sell kits that provide all of the materials listed in this protocol (e.g., Profection Mammalian Transfection System from Promega). These kits are somewhat expensive (reagent cost is about \$1 per 60-mm dish), but they serve as a useful source of control reagents when performing DEAE-dextran transfection experiments for the first time.

METHOD

1. Twenty-four hours before transfection, harvest exponentially growing cells by trypsinization and transfer them to 60-mm tissue culture dishes at a density of 10^5 cells/dish (or 35-mm dishes at a density of 5×10^4 cells/dish). Add 5 ml (or 3 ml for 35-mm dish) of complete growth medium, and incubate the cultures for 20–24 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.
The cells should be ~75% confluent at the time of transfection. If the cells are grown for <12 hours before transfection, they will not be well anchored to the substratum and are more likely to detach during exposure to DEAE-dextran.
2. Prepare the DNA/DEAE-dextran/TBS-D solution by mixing 0.1–4 µg of supercoiled or circular plasmid DNA into 1 mg/ml DEAE-dextran in TBS-D.
0.25 ml of the solution is required for each 60-mm dish; 0.15 ml is required for each 35-mm dish.
The amount of DNA required to achieve maximal levels of transient expression depends on the exact nature of the construct and should be determined in preliminary experiments. If the construct carries a replicon that will function in the transfected cells (e.g., the SV40 early region promoter/origin of replication), 100–200 ng of DNA per 10^5 cells should be sufficient; if no replicon is present, larger amounts of DNA may be required (up to 1 µg per 10^5 cells).
3. Remove the medium from the cell culture dishes by aspiration, and wash the monolayers twice with warmed (37°C) PBS and once with warmed TBS-D.
4. Add the DNA/DEAE-dextran/TBS-D solution (250 µl per 60-mm dish, 150 µl per 35-mm dish). Rock the dishes gently to spread the solution evenly across the monolayer of cells. Return the cultures to the incubator for 30–90 minutes (the time will depend on the sensitivity of each batch of cells to the DNA/DEAE-dextran/TBS-D solution). At 15–20-minute intervals, remove the dishes from the incubator, swirl them gently, and check the appearance of the cells under the microscope. If the cells are still firmly attached to the substratum, continue the incubation. Stop the incubation when the cells begin to shrink and round up.

5. Remove the DNA/DEAE-dextran/TBS-D solution by aspiration. Gently wash the monolayers once with warmed TBS-D and then once with warmed PBS, taking care not to dislodge the transfected cells.
6. Add 5 ml (per 60-mm dish) or 3 ml (per 35-mm dish) of warmed medium supplemented with serum and chloroquine (100 μ M final concentration), and incubate the cultures for 3–5 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.

The efficiency of transfection is increased severalfold by treatment with chloroquine, which may act by inhibiting the degradation of the DNA by lysosomal hydrolases (Luthman and Magnusson 1983). Note, however, that the cytotoxic effects of a combination of DEAE-dextran and chloroquine can be severe. It is therefore important to carry out preliminary experiments to determine the maximum permissible length of exposure to chloroquine after treatment of cells with DEAE-dextran (for further details, please see the information panel on **CHOROQUINE DIPHOSPHATE**).

7. Remove the medium by aspiration, and wash the monolayers three times with serum-free medium. Add to the cells 5 ml (per 60-mm dish) or 3 ml (per 35-mm dish) of medium supplemented with serum, and incubate the cultures for 36–60 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂ before assaying for transient expression of the transfected DNA.

The time of incubation should be optimized for the particular cell line and construct under study.

8. To assay the transfected cells for transient expression of the introduced DNA, harvest the cells 36–60 hours after transfection. Analyze RNA or DNA using hybridization. Analyze newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following *in vivo* metabolic labeling, or by assays of enzymatic activity in cell extracts.

To minimize dish-to-dish variation in transfection efficiency, it is best to (i) transfect several dishes with each construct, (ii) trypsinize the cells for 24 hours of incubation, (iii) pool the cells, and (iv) replat them on several dishes.

ALTERNATIVE PROTOCOL: TRANSFECTION MEDIATED BY DEAE-DEXTRAN: INCREASED CELL VIABILITY

By contrast to the DEAE-dextran method described in the main protocol, this alternative protocol uses a lower concentration of DEAE-dextran (250 µg/ml) that remains in contact with the cells for longer periods of time (up to 8 hours). Although transfection frequencies are not as high as those obtained in the presence of elevated DEAE-dextran concentrations, the use of reduced levels of DEAE-dextran is associated with less cell toxicity.

Additional Materials*Dulbecco's modified Eagle's medium*

This is standard DMEM buffered with NaHCO₃ and supplemented with serum.

Dulbecco's modified Eagle's medium, buffered with HEPES (HEPES-buffered DMEM)

This is DMEM lacking NaHCO₃ but containing 10 mM HEPES (pH 7.15). No serum should be added to this reagent.

Method

1. Twenty-four hours before transfection, harvest exponentially growing cells by trypsinization and transfer them to 60-mm tissue culture dishes with 10^5 cells/dish (or 35-mm dishes with 5×10^4 cells/dish). Add 5 ml (or 3 ml for 35-mm dish) of complete growth medium, and incubate the cultures for 20–24 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.

The cells should be ~75% confluent at the time of transfection. If the cells are grown for <12 hours before transfection, they will not be well anchored to the substratum and are more likely to detach during exposure to DEAE-dextran.

2. Mix 0.1–1 µg of supercoiled or circular plasmid DNA and 250 µg of DEAE-dextran per 1 ml of HEPES-buffered DMEM. The resulting solution will be used at 500 µl per 60-mm dish or 250 µl per 35-mm dish.

The amount of DNA required to achieve maximal levels of transient expression depends on the exact nature of the construct and should be determined in preliminary experiments. If the construct carries a replicon that will function in the transfected cells (e.g., the SV40 early region promoter/origin of replication), 100–200 ng of DNA per 10^5 cells should be sufficient; if no replicon is present, larger amounts of DNA may be required (up to 1 µg per 10^5 cells).

3. Remove the medium from the cell culture dishes by aspiration, and wash the monolayers twice with warmed (37°C) HEPES-buffered DMEM.

4. Add the DNA/DEAE-dextran/DMEM solution to the cells (500 µl per 60-mm dish, 250 µl per 35-mm dish), and return the cells to the incubator for up to 8 hours. Gently rock the dishes every 2 hours to ensure even exposure to the DNA/DEAE-dextran/DMEM solution.

The efficiency of transfection is increased severalfold by concurrent treatment of the cells with chloroquine diphosphate. If used, add the drug (100 µM final concentration) to the DNA/DEAE-dextran solution just before it is applied to the cells. Because chloroquine is toxic to the cells, the time of incubation must then be limited to 3–5 hours.

A simple variation on this step is reported to double the transfection frequency obtained with COS cells (Gonzales and Jolly 1995). Plate the cells in small culture flasks with screw caps at the beginning of the experiment and tightly screw the cap after addition of the DNA/DEAE-dextran/DMEM solution in Step 4. Continue the incubation for 8 hours, during which time the medium alkalizes slowly, due to the metabolism of the small amount of CO₂ remaining in the flask. This change, which is marked by the gradual deepening in color from crimson to burgundy of the phenol red indicator in the medium, may stimulate transfection in a manner similar to the use of a reduced CO₂ atmosphere within the incubator (please see the **ALTERNATIVE PROTOCOL: HIGH-EFFICIENCY CALCIUM-PHOSPHATE-MEDIATED TRANSFECTION OF EUKARYOTIC CELLS WITH PLASMID DNAs** in Protocol 2).

5. Remove the DNA/DEAE-dextran/DMEM solution from the cells by aspiration, and gently wash the cell monolayers twice with warmed (37°C) HEPES-buffered DMEM. Take care not to dislodge the transfected cells.

6. Wash the cells once with warmed DMEM (buffered with NaHCO₃, not HEPES) supplemented with serum. Add to the cells 5 ml (per 60-mm dish) or 3 ml (per 35-mm dish) of complete growth medium, and incubate the cultures for 36–60 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂, before assaying for transient expression of the transfected DNA.

7. To assay the transfected cells for transient expression of the introduced DNA, harvest the cells 36–60 hours after transfection. Analyze RNA or DNA using hybridization. Analyze newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following *in vivo* metabolic labeling, or by assays of enzymatic activity in cell extracts.

To minimize dish-to-dish variation in transfection efficiency, it is best to (i) transfect several dishes with each construct, (ii) trypsinize the cells after 24 hours of incubation, (iii) pool them, and (iv) replat them on several dishes.

Protocol 5

DNA Transfection by Electroporation

PULSED ELECTRICAL FIELDS CAN BE USED TO INTRODUCE DNA into a variety of animal cells (Neumann et al. 1982; Wong and Neumann 1982; Potter et al. 1984; Sugden et al. 1985; Toneguzzo et al. 1986; Tur-Kaspa et al. 1986), plant cells (Fromm et al. 1985, 1986; Ecker and Davis 1986), and bacteria. Electroporation works well with cell lines that are refractive to other techniques, such as calcium phosphate-DNA coprecipitation. But, as with other transfection methods, the optimal conditions for electroporating DNA into untested cell lines must be determined empirically.

The efficiency of transfection by electroporation is influenced by a number of factors as described below.

- **Strength of the applied electric field.** At low voltage, the plasma membranes of cultured cells are not sufficiently altered to allow passage of DNA molecules; at higher voltage, the cells are irreversibly damaged. For most lines of mammalian cells, the maximal level of transient expression is reached when voltages between 250 V/cm and 750 V/cm are applied. Typically, between 20% and 50% of the cells survive this treatment (as measured by exclusion of trypan blue [Patterson 1979; Baum et al. 1994]).
- **Length of the electric pulse.** Usually, a single electric pulse is passed through the cells. The length, field shape, and strength of the pulse are determined by the capacitance of the power supply and the dimensions of the cuvette. Some electroporation devices grant the investigator control over the characteristics of the pulse; others do not. The optimal length of the electric pulse required for electroporation is 20–100 msec.
- **Temperature.** Some investigators report that maximal levels of transient expression are obtained when the cells are maintained at room temperature during electroporation (Chu et al. 1987); others have obtained better results when the cells are maintained at 0°C (Reiss et al. 1986). These discrepancies may result from differences in the responses of various types of mammalian cells to the passage of electric current or in the amount of heat generated during electroporation when large electrical voltages (>1000 V/cm) and/or extended electric pulses (>100 msec) are used. The efficiency of transient expression is increased if the cells are incubated for 1–2 minutes in the electroporation chamber after exposure to the electric pulse (Rabussay et al. 1987).

- **Conformation and concentration of DNA.** Although both linear and circular DNAs can be transfected by electroporation, higher levels of both transient expression and stable transformation are obtained when linear DNA is used (Neumann et al. 1982; Potter et al. 1984; Toneguzzo et al. 1986). Effective transfection has been obtained with concentrations of DNA ranging from 1 $\mu\text{g}/\text{ml}$ to 40 $\mu\text{g}/\text{ml}$.
- **Ionic composition of the medium.** The efficiency of transfection is manyfold higher when the cells are suspended in buffered salt solutions (e.g., HEPES-buffered saline) rather than in buffered solutions of nonionic substances such as mannitol or sucrose (Rabussay et al. 1987).

A number of different electroporation instruments are available commercially, and the manufacturers supply detailed protocols for their use. The following method is adapted from a protocol provided by Jennifer Cuthbert and Rhonda Bassel-Duby (University of Texas Southwestern Medical Center, Dallas) and from Baum et al. (1994).

For a review of methods for the introduction of DNA molecules into eukaryotic cells by electroporation, please see Andreason and Evans (1988); for a discussion of the use of electroporation to introduce DNA into bacterial cells, please see Chapter 1, Protocol 26; and for further information on the mechanism of electroporation and optimization, please see the information panel on ELECTROPORATION.

MATERIALS

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

Giemsa stain (10% w/v)

The Giemsa stain should be freshly prepared in phosphate-buffered saline or H_2O and filtered through Whatman No. 1 filter paper before use.

Methanol <!>

Phosphate-buffered saline (PBS)

Sterilize the solution by filtration before use and store it at room temperature.

Sodium butyrate (500 mM) (optional)

In a chemical fume hood, bring an aliquot of stock butyric acid solution to a pH of 7.0 with 10 N NaOH. Sterilize the solution by passing it through a 0.22- μm filter; store the filtrate at -20°C .

Nucleic Acids and Oligonucleotides

Carrier DNA (10 mg/ml; e.g., sonicated salmon sperm DNA) (optional)

Linearized or circular plasmid DNA (1 $\mu\text{g}/\mu\text{l}$ in sterile deionized H_2O)

Media

Cell culture growth medium (complete and [optional] selective)

Centrifuges and Rotors

Sorvall H1000B rotor or equivalent

Special Equipment

Electroporation device and cuvettes

Gene Pulser II (Bio-Rad Cat. no. 165-2105 for 110-V U.S. Systems and Cat. no. 165-2106 for 220-V European Systems).

Tissue culture dishes (35-mm)

This protocol is designed for cells grown in 35-mm culture dishes. If multiwell plates, flasks, or dishes of a different diameter are used, scale the cell density and reagent volumes accordingly. Please see Table 16-3.

Additional Reagents

Step 10 of this protocol may require the reagents listed in Chapter 17, Protocol 7, or in the additional protocol in Protocol 1.

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. Harvest the cells to be transfected from cultures in the mid- to late-logarithmic phase of growth. Use either a rubber policeman or trypsin to release adherent cells. Centrifuge at 500g (1500 rpm in a Sorvall H1000B rotor) for 5 minutes at 4°C.
2. Resuspend the cell pellet in 0.5x volume of the original growth medium and measure the cell number using a hemocytometer (please see Appendix 8).
3. Collect the cells by centrifugation as described in Step 1, and resuspend them in growth medium or phosphate-buffered saline at room temperature at a concentration of 2.5×10^6 to 2.5×10^7 cells/ml.
4. Transfer 400- μ l aliquots of the cell suspension (10^6 to 10^7 cells) into as many labeled electroporation cuvettes as needed. Place the loaded cuvettes on ice.
5. Set the parameters on the electroporation device. A typical capacitance value is 1050 μ F. Voltages range from 200 to 350 V, depending on the cell line, but generally average 260 V. Use an infinite internal resistance value. Discharge a blank cuvette containing phosphate-buffered saline at least twice before beginning electroporation of cells.
6. Add 10–30 μ g of plasmid DNA in a volume of up to 40 μ l to each cuvette containing cells. (Some investigators add carrier DNA [e.g., salmon sperm DNA] to bring the total amount of DNA to 120 μ g.) Gently mix the cells and DNA by pipetting the solution up and down. Proceed to Step 7 without delay.

▲ **IMPORTANT** Do not introduce air bubbles into the suspension during the mixing step.
7. Immediately transfer the cuvette to the electroporator and discharge the device. After 1–2 minutes, remove the cuvette, place it on ice, and proceed immediately to the next step.

8. Transfer the electroporated cells to a 35-mm culture dish using a micropipettor equipped with a sterile tip. Rinse out the cuvette with a fresh aliquot of growth medium, and add the washings to the culture dish. Transfer the dish to a humidified incubator at 37°C with an atmosphere of 5–7% CO₂.

To incorporate a sodium butyrate shock (please see Protocol 2, Step 5), rinse the cuvette with growth medium containing an appropriate amount of sodium butyrate, and combine the rinse with the electroporated cells in the dish before its transfer to an incubator. After 24 hours, remove butyrate-containing medium and replace with normal growth medium.
9. Repeat Steps 6–8 until all of the DNA cell samples in cuvettes are shocked. Record the actual pulse time for each cuvette to facilitate comparisons between experiments.
10. If the objective is stable transformation of the cells, proceed directly to Step 11. For transient expression, examine the cells 24–96 hours after electroporation using one of the following assays:
 - If a plasmid DNA expressing *E. coli* β -galactosidase was used, follow the steps outlined in Chapter 17, Protocol 7 to measure enzyme activity in cell lysates. Alternatively, carry out a histochemical staining assay as detailed in the additional protocol in Protocol 1.
 - If a green fluorescence protein expression vector was used, examine the cells with a microscope under 450–490-nm illumination.
 - For other gene products, analyze the newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following *in vivo* metabolic labeling, or by assays of appropriate enzymatic activity in cell extracts.

To minimize dish-to-dish variation in transfection efficiency, it is best to (i) transfect several dishes with each construct, (ii) trypsinize the cells after 24 hours of incubation, (iii) pool the cells, and (iv) replat them on several dishes.
11. To isolate stable transfectants: After incubation for 48–72 hours in complete medium, trypsinize the cells and replat them in the appropriate selective medium. The selective medium should be changed every 2–4 days for 2–3 weeks to remove the debris of dead cells and to allow colonies of resistant cells to grow. Thereafter, clone individual colonies and propagate for assay (for methods, please see Jakoby and Pastan 1979 or Spector et al. 1998b [Chapter 86 in *Cells: A Laboratory Manual*]).

A permanent record of the numbers of colonies may be obtained by fixing the remaining cells with ice-cold methanol for 15 minutes, followed by staining with 10% Giemsa for 15 minutes at room temperature before rinsing in tap water. The Giemsa stain should be freshly prepared in phosphate-buffered saline or H₂O and filtered through Whatman No. 1 filter paper before use.

Protocol 6

DNA Transfection by Biolistics

DESPITE THE MYRIAD AND INGENIOUS METHODS developed to introduce cloned DNAs into cells, some cell types, tissues, and intracellular organelles remain impermeable to foreign DNA. This problem, which is particularly acute with plant cells, was solved to a large degree with the invention of the "gene gun" by John Sanford, Ed Wolf, and their colleagues at Cornell University. The gene gun was of greatest interest to plant geneticists, whose attempts to introduce DNA into plant cells by conventional methods had been frustratingly inefficient. The gun solved the problem by the crude but efficient strategy of drilling holes through the thick cell walls with DNA-coated metal particles. Despite the initial enormous skepticism of other scientists, Sanford and Wolf published papers on the subject (Klein et al. 1987; Shark et al. 1991; Smith et al. 1992; Sanford et al. 1993), obtained patents on the gene gun and its uses as an instrument to deliver DNA to cells, and formed a company with the apt name of Biolistics, Inc. This method has revolutionized plant genetics: Most of the world's transgenic crops have been produced using biolistic technology. The travails of Sanford and Wolf and the scientific and commercial rewards of their work are beautifully described by Sanford in a publication available from his philanthropic organization (Feed My Sheep Foundation, Waterloo, New York).

The efficiency with which foreign DNA is introduced into cells by biolistic transformation depends on a large number of variables, including:

- **Cell type.** Cells ranging from bacteria to plants to hepatocytes within the livers of living rodents have been successfully shot with DNA-coated microprojectiles. In each case, the conditions that produced a successful experiment were different.
- **Cell growth.** The density at which cultured cells are bombarded affects the transfection frequency, with greater efficiency realized at low cell density in some cases (e.g., when introducing DNA into intracellular organelles) and at high cell density in others (e.g., when cells of *Bacillus megaterium* are the target). Some cells are best transfected while in the early log phase of their growth cycle, whereas others succumb after being grown to saturation.
- **Culture medium.** The efficiencies with which many bacterial and plant cells can be transfected by biolistic methods can be increased considerably by bombarding the cells in a medium of high osmolarity. Agents such as sorbitol and/or mannitol in concentrations ranging from 0.05 to 1.5 M are used, with the optimum osmoticum and concentration differing between species and cell type.
- **Gene gun settings.** Important parameters include the amount of vacuum applied to the shooting chamber, the helium pressure used to drive the DNA-coated particles, and the distance between the gun and the target cells.

- **Type of ammunition.** Blasting subcellular organelles with inappropriately large-diameter gold or tungsten particles produces only a mess. The optimum particle size for each application differs between cell types and ranges from 0.6 μm for subcellular organelles to 1.6 μm for cultured mammalian cells. Particles made of tungsten or gold are used to deliver the DNA to cells and each metal has its idiosyncrasies. Tungsten particles are irregular in size and some cells are sensitive to the toxic effects of this transition metal. Tungsten is also susceptible to oxidation, which promotes the degradation of DNA. Gold is less toxic and more malleable and consequently can be shaped into particles that are more uniform in diameter. However, gold binds DNA less efficiently and is more expensive than tungsten.

As with many other methods of transfection, the bewildering array of variables that affect the efficiency of biolistic transformation requires that the optimum conditions for gene delivery be determined empirically in each laboratory and for each cell type. Optimization is best achieved by applying a matrix of variables in the initial series of experiments as described by Sanford et al. (1993). From these results, a reproducible method of biolistic transformation can be established for experimental purposes. The following protocol is an amalgam derived from Horch et al. (1999), Sanford et al. (1993), publications from the principal gene gun manufacturer (US/EG Bulletins 1688 and 2087; Bio-Rad), and a method contributed by Steve Finkbeiner (University of California, San Francisco).

MATERIALS

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

CaCl₂ (2.5 M)

Ethanol

Use a fresh bottle of absolute ethanol that has not been opened previously. Ethanol is hygroscopic and with exposure to air picks up small amounts of water. In the method described below, the presence of water in the ethanol washes of Steps 1, 2, and 3 can interfere with effective bombardment of cells and tissues.

Glycerol (50% in H₂O)

Sterilize the solution by autoclaving.

Spermidine (0.1 M)

Dissolve an appropriate amount of spermidine (free-base form) in deionized H₂O and sterilize the solution by passing it through a 0.22- μm filter. Store the solution in small aliquots at -20°C . Make a fresh stock solution of this reagent every month.

Nucleic Acids and Oligonucleotides

Plasmid DNA

When carrying out a gene gun experiment for the first time or if a new cell line or tissue is to be transfected, obtain an expression plasmid encoding an appropriate marker gene for use in optimizing delivery. Examples include vectors that express *E. coli* β -galactosidase, green fluorescent protein, and β -glucuronidase (for plants) or selectable markers such as neomycin resistance. Plasmids expressing a gene or cDNA of interest can be used after the process has been optimized.

Investigators debate whether plasmid DNAs purified on various chromatographic resins (please see Chapter 1, Protocol 9), which contain varying amounts of lipopolysaccharide, can be used in biolistic gene delivery. Apparently, even minor contamination of the plasmid DNA with endotoxin/lipopolysaccharide reduces the frequency of transfection. We thus recommend purification of plasmid DNAs by CsCl-ethidium bromide centrifugation (Chapter 1, Protocol 10). Dissolve the purified plasmid DNA in H₂O at a concentration of 1 $\mu\text{g}/\mu\text{l}$.

Media

Cell culture growth medium (complete and [optional] selective)

Special Equipment

Gene gun

A popular model is the Biolistic PDS-1000/He Particle Delivery System, sold by Bio-Rad, which includes a bombardment chamber with separate connections for vacuum and helium lines. A pump capable of pulling a vacuum of 5 inches of mercury is required. In general, house vacuum lines are unsuitable for this task. A high-pressure (2400–2600 psi) tank of helium gas (>99.999% pure) that is safely anchored to the bench or wall should be connected to the device.

Gold or tungsten particles (microcarriers)

The DNA to be transfected is delivered to cells on tungsten or gold particles that vary in diameter from 0.6 to 5 μm . The optimum pellet diameter for a given cell or tissue type must be determined empirically. The beads are purchased from commercial sources (e.g., Bio-Rad and Sylvania) and prepared for DNA coating as described in Step 1.

Lens paper

Microfuge tubes (1.5 ml)

Use high-quality microfuge tubes. Some brands or batches of microfuge tubes bind excessive amounts of the colloidal particles used as ammunition for gene gun experiments.

Additional Reagents

Step 8 of this protocol may require the reagents listed in Chapter 17, Protocol 7.

Cells and Tissues

Cells or tissue to be transfected

Adherent cultured cells from various species should be bombarded at 50–80% confluency. Collect plant cells grown in suspension by sterile filtration onto Whatman No. 1 filter papers (7-cm diameter) using a Buchner funnel, and place them on sterile filter papers soaked with culture medium of high osmolarity before bombardment (for details, please see Sanford et al. 1993).

Freshly dissect the mammalian tissue, section at $\sim 400 \mu\text{m}$, and maintain the slices in culture dishes as described by McAllister et al. (1995).

Culture bacteria and yeast to mid- to late-logarithmic growth depending on the species and strain, collect by centrifugation, resuspend in a small volume of culture medium of high osmolarity, and plate (1×10^8 to 2×10^9 cells) on a thin layer of agar atop a piece of filter paper in a Petri dish before being shot.

METHOD

1. Prepare tungsten or gold particles.

- a. Weigh 60 mg of gold or tungsten particles into a 1.5-ml microfuge tube.
- b. Add 1 ml of 70% ethanol to the particles and vortex the tube continuously for 5 minutes at room temperature. Store the tube on the bench top for 15 minutes.
- c. Collect the particles by centrifugation at maximum speed for 5 seconds in a microfuge.
- d. Gently remove the supernatant. Resuspend the metal particles in 1-ml of sterile H_2O and vortex the suspension for 1 minute. Store the tube on the bench top for 1 minute.
- e. Collect the metal particles by centrifugation at maximum speed for 5 seconds in a microfuge.

f. Repeat the H_2O wash (Steps d and e) three more times.

g. Remove the supernatant after the fourth H_2O wash. Resuspend the particles in 1 ml of sterile 50% glycerol.

The washed particles are assumed to have a concentration of 60 mg/ml and may be stored at room temperature for 1–2 weeks. Longer storage can result in oxidation of the metal beads and a decline in transfection efficiency.

2. For every six dishes of cells or slices of tissue to be shot, prepare an aliquot of DNA-coated particles as follows:

a. While continuously vortexing the stock solution of microcarrier particles, remove a 50- μ l aliquot (~3 mg).

b. Transfer the aliquot to a fresh microfuge tube, and while vortexing, add the following to the tube:

plasmid DNA (~2.5 μ g)	2.5 μ l
2.5 M $CaCl_2$	50 μ l
0.1 M spermidine	20 μ l

After all ingredients are added, continue vortexing the tube for an additional 3 minutes.

It is very important that the microfuge tube be continuously vortexed during this procedure to ensure uniform coating of the particles with plasmid DNA.

c. Stand the tube on the bench for 1 minute to allow the particles to settle, and then collect them by centrifugation at maximum speed for 2 seconds in a microfuge.

Longer centrifugation times can cause agglomeration of the metal particles, reducing transfection efficiency.

d. Remove the supernatant and carefully layer 140 μ l of 70% ethanol over the pelleted particles. Remove the 70% ethanol, and add 140 μ l of 100% ethanol, again without disturbing the particles. Remove the supernatant and replace with 50 μ l of ethanol.

e. Resuspend the particle pellet by tapping the side of the tube, followed by gentle vortexing for 2–3 seconds.

3. Place a macrocarrier in the metal holder of the gene gun apparatus using the seating device supplied by the manufacturer. Wash the sheet twice with 6- μ l aliquots of ethanol. Between washes, blot the sheet dry with lens paper.

4. Vortex the pellet sample from Step 2e for 1 minute. While vortexing, withdraw 6 μ l of the pellet slurry (~500 μ g of particles) and, as quickly as possible, spread the aliquot around the central 1 cm of the macrocarrier.

5. Repeat Steps 3 and 4 until the desired number of loaded macrocarriers has been prepared. Allow the ethanol solution containing the DNA-coated particles to dry on the macrocarrier.

6. Load a macrocarrier into the gene gun, and following the manufacturer's directions, shoot a plate of cells or tissue slice.

7. After the vacuum has returned to atmospheric pressure, remove the wounded cells or tissue and place in appropriate culture conditions. Remove the ruptured macrocarrier and repeat Steps 6 and 7 until all plates are shot.

8. If the objective is stable transformation of the cells, proceed directly to Step 9. For transient expression, examine the cells 24–96 hours after shooting, using one of the following assays.
 - If a plasmid DNA expressing *E. coli* β -galactosidase was used, follow the steps outlined in Chapter 17, Protocol 7, to measure enzyme activity in cell lysates. Alternatively, carry out a histochemical staining assay as detailed in the additional protocol in Protocol 1 of this chapter.
 - If a green fluorescence protein expression vector was used, examine the cells with a microscope under 450–490-nm illumination.
 - If a plasmid DNA expressing β -glucuronidase was used, assay for β -glucuronidase activity as detailed in the panel on **ADDITIONAL PROTOCOL: HISTOCHEMICAL STAINING OF CELL MONOLAYERS OR TISSUE FOR β -GLUCURONIDASE**.
 - For other gene products, analyze the newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following *in vivo* metabolic labeling, or by assays of appropriate enzymatic activity in cell extracts.

To minimize dish-to-dish variation in transfection efficiency, it is best to (i) transfect several dishes with each construct, (ii) trypsinize the cells after 24 hours of incubation, (iii) pool the cells, and (iv) replat them on several dishes.
9. To isolate stable transfectants: After the cells have incubated for 48–72 hours in complete medium, transfer the bombarded cells to selective medium. The concentration of selective agent and the culture conditions will vary depending on the cell type.

ADDITIONAL PROTOCOL: HISTOCHEMICAL STAINING OF CELL MONOLAYERS OR TISSUE FOR β -GLUCURONIDASE

β -glucuronidase is often used as a reporter gene in transfected plant cells because the endogenous levels of this hydrolase are very low. The enzyme cleaves many β -glucuronide linkages with high efficiency, including those β -glucuronidases conjugated with fluorescent and histochemical tags that are readily visualized within cells or lysates. This protocol was adapted from Jefferson et al. (1987).

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <1>.

Additional Materials

Formaldehyde (0.3% v/v) <1>

X-GlcA solution

10 mM EDTA (pH 8.0)

100 mM sodium phosphate

0.5 mM potassium ferrocyanide <1>

0.1% (v/v) Triton X-100

0.5 mg/ml X-GlcA (5-bromo-4-chloro-3-indolyl- β -D-glucuronide)

Dissolve an appropriate amount of X-GlcA (Sigma) in a small volume of DMSO before adding it to the buffer.

Mannitol (0.3 M)

MES (10 mM, pH 5.6)

NaH_2PO_4 (50 mM)

Method

1. Remove the medium in which the plant cells or tissue are maintained and replace with just enough X-GlcA solution to cover the biological material.

With some plant tissues, the intensity of β -glucuronidase staining can be enhanced by fixing the tissue before addition of the X-GlcA solution. Incubate the section in 0.3% (v/v) formaldehyde/10 mM MES (pH 5.6)/0.3 M mannitol for 30–60 minutes, rinse the section several times with 50 mM NaH_2PO_4 , and then cover it with X-GlcA staining solution.

2. Incubate the plates for 12–24 hours at 37°C.
3. Examine the bombarded cells under a light microscope and count the number of blue cells or blue clusters of cells. Cells that have taken up the β -glucuronidase expression plasmid and are synthesizing the enzyme should be a dark blue.

The number of blue spots per dish can be considered to be at best a semiquantitative estimate of transfection efficiency. This number is most useful in determining the optimum conditions for gene bombardment experiments and should not be taken as a quantitative readout of gene expression. If a quantitative measurement of gene expression is needed, then it is best to establish an *in vitro* biochemical assay using a fluorescent β -glucuronidase substrate and cell lysates prepared from transfected cells or tissue slices. For a detailed description of this type of assay, please see Jefferson et al. (1987).

Protocol 7

DNA Transfection Using Polybrene

SEVERAL POLYCATIONS, INCLUDING POLYBRENE (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) (Kawai and Nishizawa 1984; Chaney et al. 1986) and poly-L-ornithine (Bond and Wold 1987; Dong et al. 1993; Nead and McCance 1995), have been used in the presence of DMSO to facilitate DNA transfection of cells that are insensitive to transfection by other methods.

Variables that influence the efficiency of transfection by Polybrene are the concentrations of DMSO and polycation, the amount of DNA, the temperature of incubation with DMSO, and the length of time the cells are left in contact with the polycation-DNA mixture (Bond and Wold 1987; Aubin et al. 1988; Nead and McCance 1995). The original protocol, which was developed using chicken embryo fibroblasts, had optimal levels of transfection with 30% DMSO and 30 μ g of Polybrene. Optimal transfection into other cell lines requires different amounts of these reagents.

- Permanent transfection of human epidermal keratinocytes using Polybrene was found to be optimal after a 27% DMSO shock (Jiang et al. 1991).
- Murine NIH-3T3 cells required 15% DMSO (Aubin et al. 1988, 1997) for stable transfection, but 25% DMSO for optimal transient transfection.
- Human keratinocytes were transfected using 12 μ g/ml poly-L-ornithine during a 6-hour incubation followed by a 4-minute shock with 25% DMSO (Nead and McCance 1995).

The mechanism by which DMSO enhances the uptake of DNA is not known, but may involve a combination of permeabilization of the cell membrane and the osmotic shock. In some experiments using Polybrene (e.g., please see Ogawa et al. 1988), DMSO has been replaced by solutions containing 5–7% NaCl. DNA toxicity is generally not a problem in the Polybrene method of transfection. However, the linear relationship between DNA concentration and transformation efficiency breaks down when using very high concentrations of DNA (Kawai and Nishizawa 1984).

The method outlined below using Polybrene and DMSO was adapted from Aubin et al. (1997). This procedure works efficiently for stable transformation of Chinese hamster ovary and keratinocyte cells by plasmid DNA, yielding ~15-fold more transformants than calcium phosphate-DNA coprecipitation. However, there is no difference between the two methods in the efficiency of transformation of cells by high-molecular-weight DNA.

MATERIALS

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents.
Dilute stock solutions to the appropriate concentrations.

DMSO (30%) in serum-containing medium <!>

Dilute high-performance liquid chromatography (HPLC)-grade or tissue-culture-grade DMSO to a final concentration of 30% (v/v) in the cell growth medium containing serum just before use in Step 3.

Giemsa stain (10% w/v)

The Giemsa stain should be freshly prepared in phosphate-buffered saline or H₂O and filtered through Whatman No. 1 filter paper before use.

Methanol <!>

Polybrene (10 mg/ml)

Dissolve Polybrene (Aldrich) at a concentration of 10 mg/ml in H₂O and sterilize the solution by passing it through a 0.22- μ m filter. Store the solution as small aliquots (0.25-ml) at -20°C until needed. Discard aliquots after use.

Sodium butyrate (500 mM) (optional)

In a chemical fume hood, bring an aliquot of stock butyric acid solution to a pH of 7.0 with 10 N NaOH. Sterilize the solution by passing it through a 0.22- μ m filter. Store the filtrate in 1-ml aliquots at -20°C.

Nucleic Acids and Oligonucleotides

DNA to be transformed, e.g., plasmid DNA (1 μ g/ μ l) in H₂O

Media

Minimum essential medium (MEM)- α (containing 10% fetal calf serum, serum-free, and [optional] selective agents)

Special Equipment

Tissue culture dishes (90-mm)

This protocol is designed for cells grown in 90-mm culture dishes. If multiwell plates, flasks, or dishes of a different diameter are used, scale the cell density and reagent volumes accordingly. Please see Table 16-3.

Additional Reagents

Step 6 of this protocol may require the reagents listed in Chapter 17, Protocol 7, or in the additional protocol in Protocol 1.

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. Harvest exponentially growing cells (e.g., CHO cells) by trypsinization, and replate them at a density of 5×10^5 cells per 90-mm tissue culture dish in 10 ml of MEM- α containing 10% fetal calf serum. Incubate the cultures for 18–20 hours at 37°C in a humidified incubator in an atmosphere of 5–7% CO₂.

2. Replace the medium with 3 ml of warmed (37°C) medium containing serum, DNA (5 ng to 40 µg; no carrier DNA), and 30 µg of Polybrene. Mix the DNA with the medium before adding the 10 mg/ml Polybrene. Return the cells to the incubator for 6–16 hours. Gently rock the dishes every 90 minutes during the early stages of this incubation to ensure even exposure of the cells to the DNA-Polybrene mixture.
3. Remove the medium containing the DNA and Polybrene by aspiration. Add 5 ml of 30% DMSO in serum-containing medium. Gently swirl the DMSO medium around the dish to ensure even exposure of the cells to the solvent and place the dishes in the incubator.
4. After 4 minutes of incubation, remove the dishes from the incubator and immediately aspirate the DMSO solution. Wash the cells once or twice with warmed (37°C) serum-free medium, and add 10 ml of complete medium containing 10% fetal calf serum. If a sodium butyrate boost is to be included, then proceed to Step 5. If not, incubate the cultures for 48 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂. Then proceed directly to either Step 6 (to assay for transient expression) or Step 7 (to establish stable transformants).

Cells treated with DMSO are prone to detach from the dish. For this reason, the washing steps to remove the solvent-containing medium and the additions of fresh medium should be done as gently as possible, e.g., by slowly pipetting the medium against the side of the dish with each change.

5. (Optional) To facilitate the transfection of cells treated with DMSO and Polybrene:
 - a. Add 500 mM sodium butyrate directly to the growth medium to a final concentration of 2.5–10 mM.

The exact concentration of sodium butyrate added depends on the cell type and must be determined empirically.
 - b. Incubate the cells for 20–24 hours at 37°C in a humidified incubator with an atmosphere of 5–7% CO₂.
 - c. Remove the medium containing sodium butyrate, and replace it with butyrate-free medium containing 10% fetal bovine serum. Return the cells to the incubator.

Sodium butyrate treatment of DMSO-permeabilized cells can enhance the transient (but not permanent) expression from certain recombinant plasmids (Aubin et al. 1997), especially those carrying the SV40 early promoter/enhancer, in simian and human cells (Gorman et al. 1983a).
6. If the objective is stable transformation of the cells, proceed directly to Step 7. For transient expression, examine the cells 1–2 days after transfection using one of the following assays:
 - If a plasmid DNA expressing *E. coli* β-galactosidase was used, follow the steps outlined in Chapter 17, Protocol 7, to measure enzyme activity in cell lysates. Alternatively, carry out a histochemical staining assay as detailed in the additional protocol in Protocol 1.
 - If a green fluorescence protein expression vector was used, examine the cells with a microscope under 450–490-nm illumination.
 - For other gene products, analyze the newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following *in vivo* metabolic labeling, or by assays of enzymatic activity in cell extracts.

To minimize dish-to-dish variation in transfection efficiency, it is best to (i) transfect several dishes with each construct, (ii) trypsinize the cells after 24 hours of incubation, (iii) pool the cells, and (iv) replat them on several dishes.

7. To isolate stable transfectants: After the cells have incubated for 48 hours in nonselective medium (to allow expression of the transferred gene[s] to occur [Step 4]), either trypsinize or replat the cells in the appropriate selective medium or add the selective medium directly to the cells without further manipulation. Change this medium every 2–4 days for 2–3 weeks to remove the debris of dead cells and to allow colonies of resistant cells to grow.
8. Thereafter, clone individual colonies and propagate for assay (for methods, please see Jakoby and Pastan 1979 or Spector et al. 1998b [Chapter 86 of *Cells: A Laboratory Manual*]).

A permanent record of the numbers of colonies may be obtained by fixing the remaining cells with ice-cold methanol for 15 minutes, followed by staining with 10% Giemsa for 15 minutes at room temperature before rinsing in tap water. The Giemsa stain should be freshly prepared in phosphate-buffered saline or H_2O and filtered through Whatman No. 1 filter paper before use.

COTRANSFORMATION

Analysis of function and expression of transfected genes may require the stable integration of the transfected DNA into the host chromosome. After entering the cell, some of the transfected nucleic acid is transferred from the cytoplasm to the nucleus. Depending on the cell type, up to 80% of a population of cells will then express the transfected gene in a transient fashion. At some point within the first few hours after transfection, the incoming DNA undergoes a series of nonhomologous intermolecular recombination and ligation events to form a large concatemeric structure that eventually integrates into the cellular chromosome. Each transformed cell usually contains only one of these packages, which can exceed 2 Mb in size (Perucho et al. 1980). Stable cell lines can then be isolated that carry integrated copies of the transfected DNA. Transformation rates vary widely from cell type to cell type. In the best cases, ~1 cell in 10^3 in the original transfected population stably expresses a gene(s) carried by the transfected DNA.

Because the uptake, integration, and expression of DNA are relatively rare events, stable transformants are usually isolated by selection of cells that have acquired a new phenotype. Typically, this phenotype is conferred by the presence in the transfection mixture of a gene encoding antibiotic resistance. Cells transformed for a genetic marker present on one piece of DNA frequently express another genetic marker that was originally carried on a separate DNA molecule. Therefore, cells that stably express selectable (e.g., antibiotic-resistant) markers are also likely to have incorporated other DNA sequences present among the carrier DNA. This phenomenon, in which physically unlinked genes are assembled into a single integrated array and expressed in the same transformed cell, is known as cotransformation.

The first gene to be used extensively for selection in mammalian cells was a viral (herpes simplex) gene encoding thymidine kinase (TK; Wigler et al. 1977). Although many mammalian cell lines express thymidine kinase, several TK⁻ lines were created by selection for growth in the presence of 5-bromodeoxyuridine (BrdU). When transfected and stably integrated into the host genome of cell lines lacking thymidine kinase, the viral gene confers the TK⁺ phenotype, thereby allowing growth in the presence of aminopterin (for a discussion of the basis for selection, please see the information panel on **SELECTIVE AGENTS FOR STABLE TRANSFORMATION**). Thereafter, this strategy was used to introduce foreign DNA in mammalian cells by cotransfection with a plasmid encoding the *tk* gene (Perucho et al. 1980; Robins et al. 1981). The difficulties or additional efforts involved in creating *tk* mutants promoted the search for other selection schemes. Therefore, other possibilities for selection in cotransformation studies were explored, leading to the development of vectors that express bacterial proteins which confer drug resistance in mammalian cell lines. These selectable markers include, for example, aminoglycoside phosphotransferase (resistance to G418 or neomycin), hygromycin-B phosphotransferase (resistance to hygromycin-B), xanthine-guanine phosphoribosyltransferase (resistance to mycophenolic acid and aminopterin), and puromycin-N-acetyl transferase (resistance to puromycin). All have been used with considerable success to establish stably transformed lines of mammalian cells.

In addition to providing a means to introduce exogenous genes into mammalian cells in a stable manner, it is often desirable to increase the stringency of the selective conditions in order to obtain higher levels of expression of the transfected genes. This enhancement in expression can be achieved as a result of increase in copy number, or coamplification, of the target gene and the gene conferring the resistance. A target gene that has been cotransfected with (and become integrated near) a particular marker gene is highly likely to undergo amplification with the marker under selection. Thus, the amplification of the dihydrofolate reductase (*dhfr*) gene resulting from exposure to increasing levels of methotrexate has been used successfully to overexpress cotransfected foreign genes (Schimke 1984). Similarly, the gene encoding adenosine deaminase (ADA) can be amplified through the stepwise increase in concentrations of 2'-deoxycoformycin (dCF; Kaufman et al. 1986) (for further details on gene amplification, please see Stark and Wahl 1984).

For further details on the basis for selection as well as selective conditions required for these systems, please see the information panel on **SELECTIVE AGENTS FOR STABLE TRANSFORMATION**.

SELECTIVE AGENTS FOR STABLE TRANSFORMATION

Resistance to antibiotics has proven to be effective in selecting cotransformants and, in some cases, as a driver for gene amplification.

Aminopterin

- **Mode of action.** Thymidine kinase catalyzes a reaction in an alternative pathway for the synthesis of dTTP from thymidine. The enzyme is not required under normal conditions of growth, as cells typically synthesize dTTP from dCDP. However, cells grown in the presence of aminopterin (an analog of dihydrofolate) are unable to utilize the usual pathway for synthesizing dTTP, and thus require thymidine kinase to make use of the alternative pathway.
- **Selective conditions.** Cell lines lacking endogenous thymidine kinase activity are grown in a complete medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, and 3 μM glycine (HAT medium).

G418

- **Mode of action.** This aminoglycoside antibiotic, similar in structure to neomycin, gentamycin, and kanamycin, is the most commonly used selective agent in permanent transfection experiments. G418 and its relatives block protein synthesis through interference with ribosomal functions. The bacterial enzyme aminoglycoside phosphotransferase, carried on the transposon sequence Tn5, converts G418 to a nontoxic form.
- **Selective conditions.** Because each eukaryotic cell line demonstrates a different sensitivity to this antibiotic (and some are completely resistant to it), the optimum amount required to kill nontransfected cells must be established empirically for each new cell line or strain used for permanent transfection. This optimum is established by determining a killing curve for the cell line of interest. In this type of experiment, a plasmid conferring resistance to G418 (e.g., pSV2neo or pSV3neo; Southern and Berg 1982) is transfected into the cells, and plates of transfected cells are subjected to different concentrations of G418. After a 2–3-week selection period, the concentration of G418 giving rise to the largest number of viable colonies is determined by visual inspection, or better by actual counting of the colonies after staining with Giemsa or gentian violet.

Commercial preparations of G418 vary in their concentration of active antibiotic, with the average purity being ~50%. For this reason, each batch of G418 should be titrated before use in tissue culture. Despite this variation, the amount of G418 used to obtain optimal numbers of transfected colonies is constant for well-characterized cell lines. Table 16-5 gives the optimum G418 concentration ranges for use with several commonly used cell lines.

TABLE 16-5 Selective G418 Concentration Ranges

CELL LINE OR ORGANISMS	G418 CONCENTRATION ($\mu\text{g/ml}$)
Chinese hamster ovary cells	700–800
Madin-Darby canine kidney cells	500
Human epidermoid A431 cells	400
Simian CV-1 cells	500
<i>Dictyostelium</i>	10–35
Plant	10
Yeast	125–500

TABLE 16-6 Selective Concentrations of Hygromycin B

ORGANISM	INHIBITORY CONCENTRATION OF HYGROMYCIN B	REFERENCES
<i>Escherichia coli</i>	200 µg/ml	Gritz and Davies (1983)
<i>Saccharomyces cerevisiae</i>	200 µg/ml	Gritz and Davies (1983)
Mammalian cells	12–400 µg/ml, depending on the cell line	Sugden et al. (1985); Palmer et al. (1987)

Hygromycin B <1>

- **Mode of action.** Hygromycin B is an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus* (Pittenger et al. 1953). Hygromycin B inhibits protein synthesis in both prokaryotes and eukaryotes by interfering with translocation (Cabañas et al. 1978; Gonzalez et al. 1978) and causing mistranslation in vivo and in vitro (Singh et al. 1979).

A bacterial plasmid-borne gene has been identified and sequenced (Gritz and Davies 1983), which encodes a 341-amino-acid hygromycin-B phosphotransferase (Rao et al. 1983) that inactivates the antibiotic. This gene has been used as a selectable marker in *E. coli*, and chimeric genes constructed with the appropriate promoters act as dominant selectable markers in *Saccharomyces cerevisiae* (Gritz and Davies 1983; Kaster et al. 1984), mammalian cells (Santerre et al. 1984; Sugden et al. 1985), and plants (van den Elzen 1985; Waldron et al. 1985).

- **Selective conditions.** The concentrations of antibiotic required to inhibit growth of various organisms are presented in Table 16-6.

Methotrexate (MTX) <1>

- **Mode of action.** An analog of dihydrofolate, methotrexate is a powerful inhibitor of dihydrofolate reductase (DHFR), an enzyme required for purine biosynthesis. Increasing levels of methotrexate can result in amplification of the gene encoding DHFR with concomitant increase in its levels of expression. The system is therefore extremely effective for amplification of cotransfected genes (Simonsen and Levinson 1983).
- **Selective conditions.** The medium is typically supplemented with 0.01–300 µM methotrexate.

Mycophenolic Acid

- **Mode of action.** Mycophenolic acid, a weak dibasic acid with antibiotic properties, specifically inhibits inosinate (IMP) dehydrogenase, an enzyme of mammalian cells that converts IMP to xanthine monophosphate (XMP). This block to synthesis of guanosine monophosphate (GMP) can be relieved by supplying cells with xanthine and a functional *E. coli gpt* gene, which encodes an enzyme, xanthine-guanine phosphoribosyltransferase, that converts xanthine to XMP. *E. coli gpt* can therefore be used in the presence of mycophenolic acid as a dominant selectable marker for cotransformation of mammalian cells of any type (Mulligan and Berg 1981a,b). The selection can be made more efficient by the addition of aminopterin, which blocks the endogenous pathway of purine biosynthesis (for further details, please see Gorman et al. 1983b).
- **Selective conditions.** The concentration of antibiotic required to inhibit growth in mammalian cells is ~25 µg/ml.

Puromycin

- **Mode of action.** Puromycin, acting as an analog of aminoacyl tRNA, inhibits protein synthesis by causing premature chain termination. The antibiotic becomes acetylated, and thereby inactivated, by the action of puromycin-N-actyl transferase (de la Luna et al. 1988).
- **Selective conditions.** The concentrations of antibiotic required to inhibit growth of mammalian cell lines is typically in the range of 0.5–10 µg/ml; many transformed cell lines are effectively selected at 2 µg/ml.

LIPOFECTION

Lipofection is the generic name of a set of techniques used to introduce exogenous DNAs into cultured mammalian cells. Many variants of the basic method have been developed, but they all adhere to the same general principle: The DNA to be transfected is coated by a lipid, which either interacts directly with the plasma membrane of the cell (Bangham 1992) or is taken into the cell by nonreceptor-mediated endocytosis (Zhou and Huang 1994; Zabner et al. 1995), presumably as a prelude to membrane fusion in endosomes (Pinnaduwa et al. 1989; Leventis and Silvius 1990; Rose et al. 1991). However, as with other transfection techniques, only a small percentage of liposomes deliver their cargo of DNA into the nucleus (Tseng et al. 1997). As judged from microscopy, most of the DNA remains associated with the membrane compartments of the cell, where it is unavailable for transport into the cytoplasm and subsequent movement to the nucleus (Zabner et al. 1995). Nevertheless, when working at its best, lipofection can deliver DNA into cells more efficiently than precipitation with polycations such as calcium phosphate and at lower cost than electroporation.

Like other transfection techniques, lipofection is not universally successful: The efficiency of both transient expression and stable transformation by exogenously added genes varies widely from cell line to cell line. Different types of cells may show a range of quantitative responses to the same lipofection protocol. Different protocols used on the same cell line may generate results that span an extensive range. However, lipofection works very well in many situations where standard methods are notoriously inefficient, for example, transfection of primary cultures or cultures of differentiated cells (e.g., please see Thompson et al. 1999) or introduction of very high-molecular-weight DNA into standard cell lines (e.g., please see Strauss 1996). Lipofection is therefore the method of choice for introducing genes into differentiated cells *in vitro* and is the technique of first resort when older methods of transfection are inadequate.

The Chemistry of Lipofection

There are two general classes of liposomal transfection reagents: those that are anionic and those that are cationic. Transfection with anionic liposomes, which was first used in the late 1970s to deliver DNA and RNA to cells in a biologically active form, requires that the DNA be trapped in the internal aqueous space of large artificial lamellar liposomes (for reviews of this early work, please see Fraley and Papahadjopoulos 1981, 1982; Fraley et al. 1981; Straubinger and Papahadjopoulos 1983). However, the technique in its basic form never entered widespread use, perhaps because of its time-consuming nature and problems with reproducibility by investigators who were not expert in lipid chemistry.

The lipofection techniques in common use today stem from a seminal discovery by Peter Felgner that cationic lipids react spontaneously with DNA to form a unilamellar shell which can fuse with cell membranes (Felgner et al. 1987; Felgner and Ringold 1989). The formation of DNA-lipid complexes is due to ionic interactions between the head group of the lipid, which carries a strongly positive charge that neutralizes the negatively charged phosphate groups on the DNA (please see Figure 16-1).

The first generation of cationic lipids were monocationic double-chain amphiphiles with a positively charged quaternary amino head group (Duzgunes et al. 1989), linked to the lipid backbone by ether or ester linkages. Such monocationic lipids suffer from two major problems: They are toxic to many types of mammalian and insect cells, and their ability to promote transfection is restricted to a small range of cell lines (Felgner et al. 1987; Felgner and Ringold 1989). The later generations of cationic lipids are polycationic, have a far wider host range, and are considerably less toxic than their predecessors (for review, please see Gao and Huang 1993). In most cases, preparations of cationic lipids used for transfection consist of a mixture of synthetic cationic lipid and a fusogenic lipid (phosphatidylethanolamine or DOPP). Several cationic and polycationic lipids active in transfection are described in Table 16-4. Depending on the composition of the lipid mixture, the DNA to be transfected becomes incorporated either into multilamellar structures composed of alternating layers of lipid bilayer and hydrated DNA or into hexagonal columns arranged in a honeycomb structure (Labat-Moleur et al. 1996; Koltover et al. 1998). Each column or tube in the honeycomb consists of a central core of hydrated DNA molecules and a surrounding hexagonal shell of lipid monolayers. Experiments with model systems suggest that honeycomb arrangements of this type deliver DNA across lipid bilayers more efficiently than multilamellar structures.

A Plethora of Lipofectants Is Available

The same properties of lipids that facilitate the formation of transfection-competent structures with DNA also bring unwanted side effects. Chief among these are a generalized toxicity, which is manifested by cells rounding up and detaching from the dish. In addition, lipofection is vulnerable to interference by fats and lipoproteins in serum and by charged components of the extracellular matrix such as chondroitin sulfate (Felgner and Holm 1989). Systematic modifications of the cationic and neutral lipids have been made in an effort to overcome these drawbacks (e.g., please see Behr et al. 1989; Felgner et al. 1994), resulting in a wealth of effective lipofection reagents. Unfortunately, these reagents, many of which are commercially available, work with varying efficiencies with different types of cells. Although few head-to-head comparisons of efficiency are available, the companies that market these reagents for lipofection provide useful bibliographies and lists of cell lines that can be efficiently transfected with the help of their particular products. The toxicity of these compounds varies from cell line to cell line, as does *inter alia*, the optimal ratio of cationic lipids:DNA and the amount of cationic lipid that can be added to a given number of cells (e.g., please see Felgner et al. 1987; Ho et al. 1991; Ponder et al. 1991; Farhood et al. 1992; Harrison et al. 1995).

Optimizing Lipofection

In addition to the properties and chemical composition of the cationic and neutral lipids, several other variables affect the efficiency of lipofection, including:

- **Initial density of the cell culture.** Cell monolayers should be in mid-log phase and should be between 40% and 75% confluent.
- **Amount of DNA added per dish.** Depending on the concentration of the sequences of interest, as little as 50 ng and as much as 40 µg of DNA might be required to obtain maximum signal from a reporter gene.
- **Medium and serum used to grow the cells.**
- **Time of exposure of cells to the cationic lipid-DNA complex,** which varies from 0.1 to 24 hours.
- **Purity of the DNA preparation.** Wherever possible, the DNA should be dissolved in H₂O rather than buffers containing EDTA. Plasmid preparations used for lipofection should be free of bacterial lipopolysaccharides and should preferably be purified by chromatography on anion exchange resins or by CsCl-ethidium bromide equilibrium density centrifugation.

All of these variables must be optimized in order to establish optimum transfection frequencies for a target cell line.

TRANSFECTION OF MAMMALIAN CELLS WITH CALCIUM PHOSPHATE-DNA COPRECIPITATES

DNA can be introduced into many lines of cultured mammalian cells as a coprecipitate with calcium phosphate. After entering the cell by endocytosis, some of the coprecipitate escapes from endosomes or lysosomes and enters the cytoplasm, from where it is transferred to the nucleus. Depending on the cell type, up to 50% of a population of cells then express transfected genes in a transient fashion. Transformed cell lines that carry integrated copies of the transfected DNA can also be selected, although at a much lower frequency. Transformation rates vary widely from cell type to cell type. In the best cases, ~1 cell in 10^3 permanently expresses a selectable marker(s) carried by the transfected DNA.

Calcium-phosphate-mediated DNA transfection was developed by Frank Graham and Alex van der Eb (1973) as a method to introduce adenovirus and SV40 DNA into adherent cultured cells. Graham and van der Eb worked out optimal conditions for the formation of calcium phosphate-DNA coprecipitates and for subsequent exposure of cells to the coprecipitate. Their work laid the foundation for the biochemical transformation of genetically marked mouse cells by cloned DNAs (Maitland and McDougall 1977; Wigler et al. 1977); for the transient expression of cloned genes in a variety of mammalian cells (e.g., please see Gorman 1985); and for the isolation and identification of cellular oncogenes, tumor-suppressing genes, and other single-copy mammalian genes (e.g., please see Wigler et al. 1978; Perucho and Wigler 1981; Weinberg 1985; Friend et al. 1988). However, Graham and van der Eb never profited financially from their discovery. That was left to Wigler, Axel, and their colleagues who in 1983 were awarded a lucrative patent for cotransformation of unlinked segments of DNA by the calcium phosphate method (please see the information panel on **COTRANSFORMATION**).

Published procedures differ widely in the manner in which calcium phosphate-DNA coprecipitates are formed prior to their addition to cells. Some methods advise against anything but the gentlest agitation and suggest, for example, that air bubbled gently from an electric pipetting device should be used to mix the DNA and the buffered solution of calcium phosphate. Other methods advocate slow mixing during addition of the DNA solution, followed by gentle vortexing. Whatever technique is chosen, the aim should be to avoid creation of coarse precipitates that are endocytosed and processed inefficiently by cells. In addition to the speed of mixing, the following are other factors that affect the efficiency of transfection.

- **Size and concentration of the DNA.** The inclusion of high-molecular-weight genomic DNA in the coprecipitate increases the efficiency of transformation by small DNAs (e.g., plasmids) (e.g., please see Chen and Okayama 1987). Soon after transfection, the small DNAs integrate in the carrier DNA, often forming an array of head-to-tail tandems. This assemblage subsequently integrates into the chromosome of the transfected cell (Perucho and Wigler 1981).
- **Exact pH of the buffer and the concentration of calcium and phosphate ions** (Jordan et al. 1996). Some investigators make up several batches of HEPES-buffered saline over the pH range 6.90–7.15 and test each batch for the quality of the calcium phosphate-DNA precipitates and for the efficiency of transformation.
- **Use of facilitators.** Increases in the efficiency of transient expression and transformation can be achieved by exposing cells to glycerol (Parker and Stark 1979), chloroquine (Luthman and Magnusson 1983), commercially available “transfection maximizers” (e.g., please see Zhang and Kain 1996), or certain inhibitors of cysteine proteases (Coonrod et al. 1997). In general, these agents are toxic to cells, and their effects on viability and transfection efficiency vary from one type of cell to another. For example, chloroquine, an amine that prevents acidification of endosomes and lysosomes and inhibits lysosomal protease cathepsin B (Wibo and Poole 1974), improves the transfection efficiency of some types of cells and decreases the efficiency of others (Chang 1994). The optimal time, length, and intensity of treatment with facilitators must therefore be determined empirically for each cell line.

The level of transient expression is determined chiefly by the intensity of transcription from the promoter and its associated *cis*-acting control elements. In specific cases, it may be possible to increase the level of expression by exposing the transfected cells to hormones, heavy metals, or other substances that activate the appropriate cellular transcription factors. In addition, expression of genes carried on plasmids that contain the SV40 enhancer can be enhanced by treating transfected simian and human cells with sodium butyrate (Gorman et al. 1983a,b). Transfection kits, which frequently include these and other modifications to the original protocol, are available from a number of companies (please see Table 16-2).

DNA transfected as a calcium phosphate coprecipitate or with DEAE-dextran as a facilitator is mutated at a high frequency (~1% per gene) in all mammalian cells examined (Calos et al. 1983; Lebkowski et al. 1984). This effect is confined to the transfected sequences and does not affect the chromosomal DNA of the host cell (Razzaque et al. 1983). The mutations, which are predominantly base substitutions and deletions, appear to occur shortly after the transfecting DNA arrives in the nucleus (Lebkowski et al. 1984). However, replication of the incoming DNA is not necessary. Because almost all of the base substitutions occur at G:C base pairs, it seems likely that the major premutational events are hydrolysis of the sugar base glycosyl bond of deoxyguanosine residues and deamination of cytosine residues. Both of these reactions occur readily at acid pH and would take place as the incoming DNA passes through endosomes, which maintain a pH of ~5 (de Duve et al. 1974). Linear DNA is especially prone to deletions (Razzaque et al. 1983; Miller et al. 1984) presumably because it serves as an attractive substrate for exonucleases. Although these mutation rates are extraordinarily high, they have little relevance to transient expression of transfected genes unless the gene of interest is large and/or has a very high content of G+C. Most of the work on mutation rates was carried out with *lacI*, which is encoded by a 750-bp segment of DNA. A gene that is 10 kb in length might therefore be expected to suffer a mutation rate of 12% or more depending on its content of G+C.

CHLOROQUINE DIPHOSPHATE

Chloroquine (FW = 519.5), an amine that prevents acidification of endosomes and lysosomes and inhibits lysosomal protease cathepsin B (Wibo and Poole 1974), increases the efficiency of transfection of some types of cells and decreases the efficiency of others (Chang 1994). By inhibiting acidification of lysosomes, chloroquine may prevent or delay the degradation of transfecting DNA by lysosomal hydrolases (Luthman and Magnusson 1983). Unfortunately, the beneficial effects of chloroquine are modest and do not extend to all cell lines. In fact, although treatment with chloroquine improves the transfection efficiency of some types of cells, it decreases the efficiency of others (Chang 1994). Because the balance between the benefits and disadvantages of chloroquine varies so widely from cell line to cell line, there is simply no way to predict whether the drug will lead to a useful increase in transfection frequency in a particular circumstance. However, if low frequencies of transfection are a problem, it is certainly worth exploring whether chloroquine can help. The optimal time, length, and intensity of treatment must be determined empirically for each cell line. Typically, however, cells will be exposed to chloroquine diphosphate at a final concentration of 100 μ M for 3–5 hours either before, during, or after the cells are exposed to a calcium phosphate–DNA coprecipitate, or during exposure of cells to a mixture of DNA and DEAE-dextran. In the presence of chloroquine, the cells develop a vesicularized appearance. After the treatment, the cells are washed with phosphate-buffered saline and medium and then incubated for 24–60 hours before assaying for expression of the transfected DNA. Chloroquine diphosphate is prepared as a 100-mM stock solution (52 mg/ml in H₂O), which should be sterilized by filtration and stored in foil-covered tubes at –20°C.

ELECTROPORATION

Nucleic acids do not enter cells under their own power; they require assistance in crossing physical barriers at the cell boundary and in reaching an intracellular site where they can be expressed and/or replicated. Exposure of many types of cells to an electrical discharge reversibly destabilizes their membranes and transiently induces the formation of aqueous pathways or membrane pores (Neumann and Rosenheck 1972; Neumann et al. 1982; Wong and Neumann 1982; for reviews, please see Zimmermann 1982; Andreason and Evans 1988; Tsong 1991; Weaver 1993) that potentiate the entry of DNA molecules (Neumann et al. 1982). This method, which is known as electroporation, has been developed into a rapid, simple, and efficient technique for introducing DNA into a wide variety of cells, including bacteria, yeasts, plant cells, and a large number of cultured mammalian cell lines. The chief practical advantages of electroporation are that it can be applied to a wide variety of cells, both prokaryotic and eukaryotic, and that it is extremely simple to carry out.

The Mechanism of Electroporation

Because the changes in membrane structure that accompany electroporation cannot be visualized in real time by microscopy, our understanding of the mechanism is based on evidence that is both patchy and circumstantial. While evidence for many of the steps is lacking, the following model (Weaver 1993) nevertheless provides a plausible account of the sequence of events that are initiated by increasing the transmembrane voltage from its physiological value of ~ 0.1 to 0.5 – 1.0 V. Figure 16-4 shows the following sequence of events:

- **The onset of electroporation causes a membrane dimple** followed by formation of transient hydrophobic pores whose diameter fluctuates from a minimum of 2 nm to a maximum of several nanometers.
- **Some of the larger hydrophobic pores are converted to hydrophilic pores** because the energy needed to form an aqueous pore is reduced as the transmembrane voltage is increased and the energy required to maintain the circumference of a large hydrophilic pore is significantly lower than that required to maintain a large hydrophobic pore. For this reason, hydrophilic pores have an extended half-life and may be further stabilized by attachment to underlying cytoskeletal elements. The generation of such long-lived metastable pores allows small ions and molecules to enter and leave the cell long after the transmembrane voltage has returned to low values (Rosenheck et al. 1975; Zimmermann et al. 1976; Lindner et al. 1977). The detailed mechanism by which molecules pass through hydrophilic pores is not known, but may include electrophoresis (Chernodnick et al. 1990) electroendo-osmosis, diffusion, and endocytosis (Weaver and Barnett 1992). Reclosing of the pores appears to be a stochastic process that can be delayed by keeping the cells at 0°C . While the pores remain open, up to 0.5 pg of DNA can enter the cell (Bertling et al. 1987). Size seems to be no impediment since DNA molecules up to 150 kb in size can easily pass through the pores (Knutson and Yee 1987). Because the DNA enters directly into the cytoplasm, it is not exposed to acid conditions in endosomes and lysosomes. This route may explain why the rate of mutation in DNA introduced to mammalian cells by electroporation is apparently very low (Drinkwater and Kleindienst 1986; Bertling et al. 1987) compared with DNA transfected as calcium phosphate coprecipitates or DEAE-dextran complexes (e.g., please see Calos et al. 1983). For *E. coli*, electroporation is currently the most efficient method available for transformation with plasmids. In excess of 80% of the cells in a culture can be transformed to ampicillin resistance by this method and efficiencies of transformation approaching the theoretical maximum of one transformant per molecule of plasmid DNA have been reported (Smith et al. 1990). However, the number of transformants obtained is marker-dependent. When pBR322, which carries genes conferring resistance to two antibiotics (ampicillin and tetracycline), is introduced into *E. coli* by electroporation, the number of tetracycline-resistant transformants is ~ 100 -fold less than the number of ampicillin-resistant transformants (Steele et al. 1994). This effect is not seen when the plasmid is introduced to the bacteria by the calcium chloride method. One possible explanation is that electroporation damages or changes the bacterial membrane so that it can no longer interact efficiently with the tetracycline resistance protein.

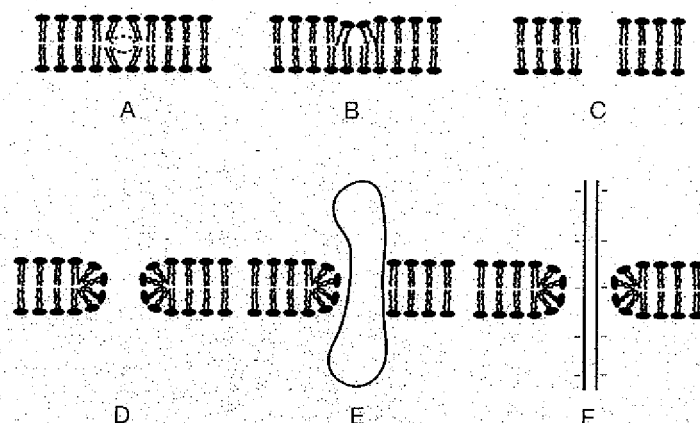


FIGURE 16-4 Changes in the Membrane during Electroporation

Drawings of hypothetical structures for transient and metastable membrane conformations believed to be relevant to electroporation. (A) Fredd volume fluctuation; (B) aqueous protrusion or "dimple"; (C,D) hydrophobic pores usually regarded as the "primary pores" through which ions and molecules pass; (E) composite pore with "foot in the door" charged macromolecule inserted into a hydrophilic pore. The transient aqueous pore model assumes that transitions from A→B→C or D occur with increasing frequency as U is increased. Type F may form by entry of a tethered macromolecule, while the transmembrane voltage is significantly elevated, and then persist after U has decayed to a small value through pore conduction. It is emphasized that these hypothetical structures have not been directly observed and that support for them derives from the interpretation of a variety of experiments involving electrical, optical, mechanical, and molecular transport behavior. (Redrawn, with permission, from Weaver 1993 [copyright Wiley-Liss, Inc.])

Typically, between 50% and 70% of cells exposed to high electric field strengths are killed. The lethal effects, which vary in intensity from one cell type to another, are not due to heating or electrolysis and are independent of the current density and energy input. Instead, cell killing is dependent on field strength and the total time of treatment (Sale and Hamilton 1967). The most likely cause of cell killing is the rupture of cell membranes, which leads to rapid loss of ionic balance and massive efflux of cellular components.

Electrical Conditions Required for Electroporation

Electroporation of almost all mammalian cells is induced when the transmembrane voltage, $\Delta U(t)$, is increased to 0.5–1.0 V for durations of microseconds to milliseconds. This translates to an electric field strength of ~7.5–15.0 kV/cm. Because this value is constant and is independent of the biochemical nature of the cell membrane, it seems likely that variations in the efficiency of electroporation from cell line to cell line are due to differences in the rate and efficiency of membrane recovery at the end of the pulse.

The transmembrane voltage, $\Delta U(t)$, induced by electric fields varies in direct proportion to the diameter of the cell that is the target for transfection (Knutson and Yee 1987). Electroporation of mammalian cells, for example, requires smaller electric fields (<10 kV/cm) than does electroporation of yeasts or bacteria (12.5–16.5 kV/cm). Most of the commercial suppliers of electroporation machines provide literature describing the approximate voltages required for transfection of specific types of cells in their particular apparatus.

Three important characteristics of the pulse affect the efficiency of electroporation: the *length* of the pulse, its *field strength*, and its *shape*. Most of the commercial electroporation machines use capacitive discharge to produce controlled pulses whose length is mainly determined by the value of the capacitor and the conductivity of the medium. Thus, the time constant of the pulse can be altered by switching capacitors according to the manufacturer's instructions or by changing the ionic strength of the medium. When the

charge from the capacitor is directed to a sample placed between two electrodes, the voltage across the electrodes rises rapidly to a peak (V_0) and declines over time (t) according to the equation:

$$V_t = V_0 [e^{-t/\tau}]$$

where t is the time constant, which is equal to the time over which the voltage declines to ~37% of peak value. τ (measured in seconds) is also equal to the product of the resistance (R , measured in ohms [Ω]) and the capacitance (C , in Farads [F]):

$$\tau = RC$$

From this equation, it follows (1) that a larger capacitor requires more time to discharge through a medium of a given resistance and (2) that a capacitor of a given size discharges more slowly as the resistance of the medium increases. Electroporation of mammalian cells is usually carried out in buffered saline solutions or culture media using a capacitor of 25.0 μF whose discharge has a time constant of ~0.5 msec.

The field strength (E) of the pulse varies in direct proportion to the applied voltage (V) and in inverse proportion to the distance (d) between the two electrodes, which is usually determined by the size of the cuvette through which the pulse travels.

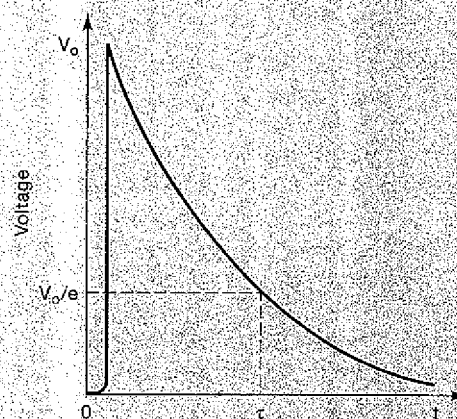
$$E = F(V, d)$$

Most manufacturers provide cuvettes in three sizes, where the interelectrode distances are, respectively, 0.1 cm, 0.2 cm, and 0.4 cm. When 1000 V are discharged into these cuvettes, E_0 is 10,000 V/cm in the 0.1-cm cuvette, 5000 V/cm in the 0.2-cm cuvette, and 2500 V/cm in the 0.4-cm cuvette.

The shape of the pulse is determined by the design of the electroporation device. The wave form produced by most commercial machines is simply the exponential decay pattern of a discharging capacitor. In some types of electroporation apparatuses, square waves can be generated by rapidly increasing the voltage, maintaining it at the desired level for a specified time (pulse width), and then rapidly reducing the voltage to zero. Square pulses can be grouped into two general categories: very high field strength of very short duration (typically 8 kV/cm for 5.4 msec) (Neumann et al. 1982), and low field strength of medium to long duration (e.g., <2 kV/cm for >10 msec) (e.g., please see Potter et al. 1984). Although differences in the effectiveness of these various waveforms have been reported from time to time (e.g., please see Knutson and Yee 1987), there is no evidence that any one of them is consistently better than any other. For all practical purposes, the exponential wave forms produced by commercial electroporation machines are perfectly satisfactory.

FIGURE 16-5 Changes in the Electric Field during Electroporation

Time course of the electric field generated by capacitor discharge. V_0 represents the time constant for the decay of the electric field; (τ) RC , where C is the capacitance of the discharge capacitor and R is the resistance of the discharge unit; the resistance of the sample cell usually determines the circuit resistance. (t) Time.



Optimizing Conditions for Electroporation

A major advantage of electroporation over other methods of transfection is that it works for a very wide variety of mammalian cells, including those that are difficult to transfect by other means (e.g., please see Potter et al. 1984; Tur-Kaspa et al. 1986; Chu et al. 1987). However, despite its advantages, electroporation is not always the most efficient way to introduce DNA into a particular cell line. For example, COS cells are transfected most efficiently by the DEAE-dextran/DNA method (Kluxen and Lübbert 1993), whereas for other cell lines, lipofection or polybrene treatment are the techniques of choice (e.g., please see Jiang et al. 1991). To find out whether electroporation is a useful method of transfection for a particular cell line, it is important to use a range of field strengths and pulse lengths and thereby to establish conditions that generate the maximum numbers of transfectants. Such conditions have been reported for >50 types of mammalian cells, and it is sometimes possible to save a lot of work by simply reading the relevant literature. Most of the companies that sell electroporation devices produce excellent up-to-date lists of papers in which electroporation has been used for transfection. These bibliographies are often the easiest way to gain access to information about the properties of a particular cell line or its close relatives. However, because of variation in properties between different cultivars of the same cell line, it is important for investigators to confirm that the conditions described in the literature are optimal for cells grown in their laboratory.

Because transfection and cell killing are independently determined by field strength (Chu et al. 1987), it is best to expose aliquots of cells to electric fields of increasing strength with time constants between 50 and 200 msec. For each field strength, measure (1) the number of cells that express a transfected reporter gene (10–40 µg/ml of linearized plasmid DNA in the electroporation buffer) and (2) the proportion of cells that survive exposure to the electric field. Plating efficiency is a more accurate measure of cell survival than staining with vital dyes since, after electroporation, cells can remain permeable to vital dyes such as trypan blue for an hour or two. The following are other variables that have been reported to affect the efficiency of electroporation.

- **The temperature of the cells before, during, and after electroporation** (e.g., please see Potter et al. 1984; Chu et al. 1987). Usually, electroporation is carried out on cells that have been prechilled to 0°C. The cells are held at 0°C after electroporation (to maintain the pores in an open position) and are diluted into warm medium for plating (Rabussay et al. 1987).
- **The concentration and conformation of the DNA** (e.g., please see Neumann et al. 1982; Potter et al. 1984; Tonneguzzo and Keating 1986). Linear DNA is preferred for stable transformation; circular DNA is for transient transfection. Preparations containing DNA at a concentration of 1 µg/ml to 80 µg/ml are optimal.
- **The state of the cells.** The best results are obtained with cell cultures in the mid-log phase of growth that are actively dividing.

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Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor.

Wigler M, Pellicer A, Silverstein S, Axel R.

Previous studies from our laboratories have demonstrated the feasibility of transferring the thymidine kinase (tk) gene from restriction endonuclease-generated fragments of herpes simplex virus (HSV) DNA to cultured mammalian cells. In this study, high molecular weight DNA from cells containing only one copy of the HSV gene coding for tk was successfully used to transform L+K-cells to the tk+ phenotype. The acquired phenotype was demonstrated to be donor-derived by analysis of the electrophoretic mobility of the tk activity, and the presence of HSV DNA sequences in the recipient cells was demonstrated. In companion experiments, we used high molecular weight DNA derived from tissues and cultured cells of a variety of species to transfer tk activity. The tk+ mouse cells transformed with human DNA were shown to express human type tk activity as determined by isoelectric focusing.

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The transfer and stable integration of the HSV thymidine kinase gene into mouse cells.

Pellicer A, Wigler M, Axel R, Silverstein S.

Treatment of mutant mouse cells (Ltk-) deficient in thymidine kinase with Bam I restriction endonuclease-cleaved HSV-1 DNA results in the appearance of numerous surviving colonies which stably express the tk+ phenotype. Through a series of electrophoretic fractionations in concert with transfection assays, we isolated a 3.4 kb fragment which contains the thymidine kinase gene and which alone is competent in the biochemical transformation of Ltk- cells. In this report, we have examined the distribution of tk sequences in the DNA of several transformed clones following stable gene transfer. A series of complementary experiments involving reassociation kinetics in solution and annealings with tk DNA to restriction-cleaved cellular DNA following electrophoresis and transfer to filters allow us to make the following general conclusions concerning the fate of the tk gene in all clones examined: the tk gene is present in all cells at a frequency of one copy per chromosomal complement; the tk gene is stably integrated in the DNA of all transformants; and integration is not site-specific and occurs at different loci in the DNA of all transformants examined. The existence of a single active tk gene in tk+ transformants now facilitates an analysis of the sequence organization of tk- mutant cells and provides a useful model system for studies on the transfer of cellular genes.

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Altering genotype and phenotype by DNA-mediated gene transfer.

Pellicer A, Robins D, Wold B, Sweet R, Jackson J, Lowy I, Roberts JM, Sim GK, Silverstein S, Axel R.

Transformation, or DNA-mediated gene transfer, permits the introduction of new genetic information into a cell and frequently results in a change in phenotype. The transforming DNA is ultimately integrated into a recipient cell chromosome. No unique chromosomal locations are apparent, different lines contain the transforming DNA on different chromosomes. Expression of transformed genes frequently results in the synthesis of new polypeptide products which restore appropriate mutant cells to the wild-type phenotype. Thus transformation provides an in vivo assay for the functional role of DNA sequence organization about specific genes. Transforming genes coding for selectable functions, such as adenine phosphoribosyltransferase or thymidine kinase, have now been isolated by utilizing transformation in concert with molecular cloning. Finally, transformation may provide a general approach to the analysis of complex heritable phenotypes by permitting the distinction between phenotypic changes without concomitant changes in DNA and functional genetic rearrangements.

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Comparison of phenotypic expression with genotypic transformation by using cloned, selectable markers. [Cell Biol. 1982]

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High efficiency DNA-mediated transformation of primate cells.

Gorman C, Padmanabhan R, Howard BH.

Tissue culture cells from several mammalian species, including three primate lines, were transfected with recombinant vectors carrying Escherichia coli xanthine-guanine phosphoribosyltransferase or Tn5 aminoglycoside phosphotransferase dominant selectable markers. Human HeLa and SV40-transformed xeroderma pigmentosum cells exhibited stable transformation frequencies of at least 10^{-3} (0.1 percent). CV-1, an African green monkey kidney cell line, could be stably transformed with the exceptionally high frequency of 6×10^{-2} (6 percent).

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Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. [Proc Natl Acad Sci USA. 1981]

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High efficiency gene transfer into mammalian cells.

Gorman CM, Lane DP, Rigby PW.

We have generalized the protocol of gene transfer, greatly increasing the variety of cells that can be used as recipients of foreign genes. Our approach has been to use a transient assay system that allows rapid screening of expression of foreign DNA. When the initial steps of gene transfer have been optimized with the transient system, these defined conditions are used to yield efficient stable transformation. We have seen that primate cells, including human cells, can be used in gene transfer experiments at levels sensitive enough to allow detection of single copy gene function. Recently we have also used this approach successfully with undifferentiated embryonic cells.

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Cotransfer of linked eukaryotic genes and efficient transfer of hypoxanthine phosphoribosyltransferase by DNA-mediated gene transfer

(thymidine kinase/galactokinase/somatic cell genetics/DNA-mediated transformation)

JANE L. PETERSON AND O. WESLEY MCBRIDE

Laboratory of Biochemistry, Division of Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Communicated by Maxine F. Singer, December 20, 1979

ABSTRACT The efficiency of DNA-mediated transfer of the gene (*hprt*) for hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) is dependent upon the recipient cell used. *hprt* has been transferred into mouse TGS or Chinese hamster CHTG49 cells at a high frequency, similar to the frequency of the gene (*tk*) for thymidine kinase (TK; ATP:thymidine 5'-phosphotransferase, EC 2.7.1.21) transfer into mouse LMTK⁻ cells (i.e., 10^{-6}). In contrast, the frequency of transfer of *hprt* into mouse A₉ cells was about two orders of magnitude less. The identification of efficient recipient cells for *hprt* transfer permits the use of DNA-mediated transfer as a bioassay for the gene. Cotransfer of the linked *tk* gene and the gene (*galk*) for galactokinase (ATP:D-galactose 1-phosphotransferase, EC 2.7.1.6) to LMTK⁻ cells has been detected once among 87 *tk* transferents. This suggests that the distance between the *tk* and *galk* genes in the Chinese hamster genome may be smaller than was previously thought. Significant differences between chromosome-mediated and DNA-mediated gene transfer were observed with respect to both the size of the transferred functional genetic fragment and the recipient cell specificity.

Transfection of eukaryotic cells with viral nucleic acids is a long-established procedure (1, 2). The frequency and reproducibility of DNA transfer were greatly enhanced by the calcium phosphate transfer technique introduced by Graham and van der Eb (3). Wigler *et al.* (4) subsequently demonstrated that single-copy eukaryotic genes can also be transferred to mammalian cells by using unfractionated total cellular DNA. They successfully transferred the cellular thymidine kinase (TK; ATP:thymidine 5'-phosphotransferase EC 2.7.1.21) gene (*tk*) from a variety of vertebrate donor species into mouse tk⁻ cells at a frequency of 1-7 transformants per 10^6 recipient cells per 20 μ g of total genomic donor DNA. DNA-mediated transformation has several obvious potential uses such as providing a convenient and sensitive bioassay for dominant-acting genes in unfractionated DNA for which no nucleic acid probes are currently available (5).

The optimal utilization of this technique requires additional information relating to both the generality of the transformation process and the size of the transferred genetic fragments. Transformation is clearly not restricted to the *tk* gene because Wigler *et al.* (5) recently reported that mouse cells deficient in adenine phosphoribosyltransferase (APRT; AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) could be transformed to the APRT⁺ phenotype at frequencies similar to the frequency for *tk* transfer. However, there has been only one report (6) of transfer of *hprt*, the gene for hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8), and it was a single in-

traspecies mouse DNA transfer at the low frequency of 0.5×10^{-7} . Conditions have been established in the present study that permit DNA transformation with *hprt* at a frequency similar to that observed for transformation with *tk* in parallel experiments (i.e., about 10^{-6}). The most important factor in achieving a high frequency for *hprt* transfer is the selection of recipient cell lines. The frequency of *hprt* transformation is at least an order of magnitude greater with Chinese hamster fibroblasts (CHTG49) or mouse 3T6 (TG8) cells than it is with mouse A₉ cells as recipients.

The size of the functional genetic fragment transferred by DNA-mediated transformation has been assessed by determining the frequency of cotransfer of two closely linked genetic loci, *tk* and *galk*, the gene for galactokinase (GalK; ATP:D-galactose 1-phosphotransferase, EC 2.7.1.6). Cotransfer of these two genes was detected only once on examination of 87 clones selected for DNA-mediated transfer of *tk*. The ability to detect cotransfer of *tk* and *galk* by transformation even at a low frequency is very surprising; it suggests that these two loci may be more closely linked than was previously thought.

MATERIALS AND METHODS

Cell Culture. Recipient cells lacking cytoplasmic TK were mouse LMTK⁻ clone 1D (7) and B₃₂ (8); those lacking HPRT were L cell A₉ (8), TG8 (a mouse 3T6 variant provided by Howard Green), and Chinese hamster TG49 (9). Culture conditions and media have been described (10, 11). Human HeLa S3, mouse L₉₂₉, and Chinese hamster fibroblasts (V-79) used as sources of donor DNA were grown in suspension cultures.

Transformation Conditions. DNA was prepared according to the method described by Pellicer *et al.* (12). In most experiments, DNA transformation of tk⁻ and *hprt*⁻ cells was carried out as described by Wigler *et al.* (4). One milliliter of calcium phosphate-precipitated DNA (20 μ g/ml) was added to 10 ml of minimal essential medium in a 100-mm plate containing 10^6 cells and incubated for 4 hr at 37°C. In a few experiments, 1 ml of the DNA/calcium phosphate precipitate was added directly to the cell monolayer as described by Graham and van der Eb (3) and incubated for 30 min before addition of 10 ml of medium and incubation for an additional 3.5 hr (37°C). Cells were then treated with 7% (vol/vol) dimethyl sulfoxide (Me₂SO) by adding 3 ml of 30% Me₂SO directly to the medium containing the precipitated DNA (13). The cells were incubated at 37°C for 30 min, the DNA and medium were removed, and fresh medium was added. Selective HAT medium containing

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; TK, thymidine kinase; GalK, galactokinase; APRT, adenine phosphoribosyltransferase; *hprt*, *tk*, *galk*, *aprt*, genes directing synthesis of HPRT, TK, GalK, and APRT, respectively; HAT, hypoxanthine/aminopterin/thymidine; Me₂SO, dimethyl sulfoxide; BrdUrd, 5-bromodeoxyuridine.

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100 μ M hypoxanthine, 1 μ M amethopterin, 16 μ M thymidine, and 100 μ M glycine (14) was added 20 hr after removal of the DNA. The medium was changed every 4 days and clones were isolated as they appeared, usually within 10 days to 2 weeks. After 4 weeks the remaining plates were fixed and stained with crystal violet to detect any additional colonies.

Preparation of Cell Extracts. Cell extracts were prepared from washed cells (approximately 10^8 cells per ml) as follows: HPRT⁺ cells were resuspended in 1 mM dithiothreitol/10 mM Tris-HCl (pH 7.4)/0.1% Triton X-100/10% (vol/vol) glycerol; TK⁺ cells were resuspended in the same buffer with the addition of 20 μ M thymidine. The cells were lysed by passage through a 26-gauge needle and the cell debris was removed by centrifugation in a Beckman Microfuge for 10 min. Between 10 and 15 μ l of extract was used for nondenaturing acrylamide gel electrophoresis and 35 μ l was used for starch gel electrophoresis.

Gel Electrophoresis. Electrophoresis of GalK as well as rodent and human HPRT was performed at 4°C in 12% starch gels at 150 V in a pH 6.8 citrate/phosphate buffer system (15). Chinese hamster and mouse HPRT were resolved by discontinuous polyacrylamide slab gel electrophoresis (10). Human and rodent TK species were separated by disc electrophoresis (16) on polyacrylamide slab gels. After electrophoresis, all gels were incubated directly with reaction mixtures containing [¹⁴C]hypoxanthine or [¹⁴C]thymidine and the phosphorylated reaction products were precipitated with LaCl₃ (17). The gels were saturated with diphenyloxazole by immersion of the polyacrylamide gels in diphenyloxazole/Me₂SO (18) and starch gels in diphenyloxazole/methanol (19) prior to dehydration and fluorography employing Kodak X-Omat film.

RESULTS

DNA-Mediated Transfer of *tk* and *hprt*. The DNA transfer methods described by Wigler *et al.* (4) and Graham and van der Eb (3) were used to stably transfer cellular *tk* and *hprt* into several cell lines. A comparison of the transfer efficiencies of the two methods is shown in Table 1, experiment 5. In this experiment, the cellular *tk* gene was transferred into mouse cells with similar efficiency by both methods of DNA addition. In other experiments (not shown), there was a slightly greater efficiency of transfer with method A than with method B. Cellular *hprt* was transferred into A₉ cells with such a low frequency ($<0.25 \times 10^{-7}$) that no conclusion about the effect of the method of DNA addition was possible. The method described by Wigler *et al.* (4) was used in all subsequent experiments.

Miller and Ruddle (13) have reported a 10-fold increase in the frequency of chromosome-mediated gene transfer in cells treated with 10% (vol/vol) Me₂SO after exposure to metaphase chromosomes. In an effort to improve the frequency of DNA-mediated transfer of the *hprt* gene into A₉ cells, cells were treated with 7% Me₂SO after exposure to DNA (Table 1, experiment 6). The Me₂SO treatment resulted in approximately a 2- to 5-fold increase in the transfer frequency of *tk* into LMTK⁻ cells or *hprt* into TG8 cells but no apparent improvement in the frequency of transfer of *hprt* into A₉ cells. The Me₂SO treatment of the cells was used in all subsequent DNA transfers.

A significant difference in the frequency of transfer of *tk* or *hprt* into other recipient cell lines was related to the recipient cell used (Table 1). For example, the frequency of transfer of *hprt* into A₉ cells was $<0.25 \times 10^{-7}$, but the transfer of *hprt* into TG8 or CHTG49 cells ranged from 0.3 to 1×10^{-6} , about the same as that for the transfer of *tk* into LMTK⁻ cells. Thus one might expect similar cell lines lacking different genes to be

Table 1. Transfer of *tk* or *hprt* gene by purified DNA

Cells/DNA	Exp.	DNA added *	Me ₂ SO	+ plates/ total†	Total colonies†
<i>tk</i> transfer					
LMTK ⁻ /CHV79	5	A	—	7/10	12
	5	B	—	3/10	3
	6	A	—	2/10	2
	6	A	+	6/10	10
	7	A	+	10/10	12
	8	A	+	7/10	8
	5	A	—	0/10	0
	5	B	—	1/10	1
LMTK ⁻ /HeLa	6	A	—	5/10	10
	6	A	+	10/10	30
	7	A	+	2/10	2
	8	A	+	5/10	5
	5	A	—	2/10	3
	5	B	—	3/10†	4†
	5	A	—	1/10†	1†
	5	B	—	1/10†	1†
<i>hprt</i> transfer					
A ₉ /CHV79	5	A	—	0/10	0
	5	B	—	1/10	1
	6	A	—	0/10	0
	6	A	+	0/10	0
A ₉ /HeLa	5	A	—	0/10	0
	5	B	—	0/10	0
	6	A	—	0/10	0
	6	A	+	1/10	1
	9§	A	+	0/10	0
	6	A	—	1/7	3
TG8/CHV79	6	A	+	10/10	19†
	9§	A	+	1/10	≥3
	9§	A	+	4/10	≥4
TG8/HeLa	7	A	+	3/10	4
	8	A	+	3/10	3
CHTG49/HeLa	8	A	+	5/10	7

* The designation A indicates that 1 ml of calcium phosphate-precipitated DNA was added to 10 ml of medium overlying the cell monolayer and incubated for 4 hr at 37°C. In method B, 1 ml of calcium phosphate-precipitated DNA was added directly to the cell monolayer and allowed to incubate 30 min before 10 ml of medium was added to the plate for 3.5 hr of additional incubation.

† All reported colonies were capable of continuous sustained growth in HAT medium.

‡ Includes a colony that was amethopterin resistant and contained no TK activity.

§ Experiment 9 was terminated at 3 weeks due to microbial contamination. Revertants cannot be excluded because cell extracts could not be prepared.

¶ Includes one revertant colony with the recipient mouse species of HPRT.

transformed at about the same frequency. However, a single experiment using the closely related B₈₂ and A₉ cells as recipients (Table 1, experiment 5) did not confirm this interpretation.

Identification of Donor Species of TK and HPRT in Transformants. To establish that the clones isolated from the DNA transfers described above were true transferrants, gel electrophoresis was performed to differentiate the enzyme species of the recipient cell from that of the donor cell. Fig. 1 *Left* and *Right* demonstrates the resolution of mouse and human TK and mouse and Chinese hamster HPRT species, respectively. Human and mouse HPRT were resolved by starch gel electrophoresis (data not shown). Table 2 summarizes these results. No system was available to separate the two rodent

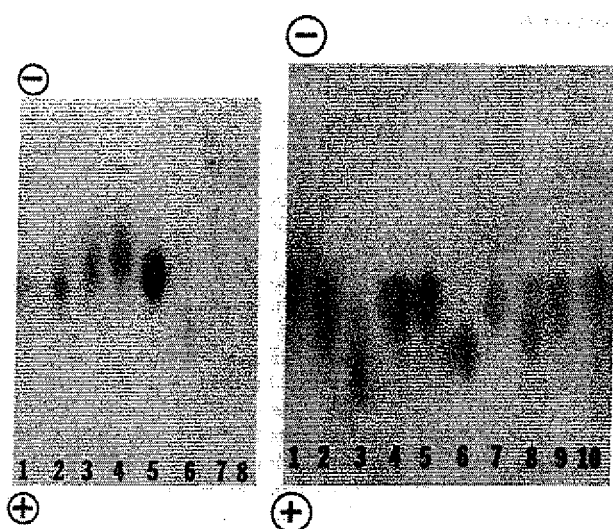


FIG. 1. (Left) Nondenaturing polyacrylamide slab gel electrophoresis of TK activity. The extracts depicted are: 5, HeLa; 6, mouse L₉₂₉; 1-4, 7, and 8, independent clones of LMTK⁻ cells transformed with HeLa DNA. (Right) Nondenaturing polyacrylamide slab gel electrophoresis of HPRT activity. The extracts are: 5, CHV79; 6, L₉₂₉; 1-4 and 7-10, extracts from independent HPRT⁺ transferrent clones (TG8 cells transformed with CHV79 DNA). A presumed revertant extract is shown in slot 3.

species of TK. Because the reversion rate for LMTK⁻ is extremely low (i.e., no revertants were found among 5×10^8 cells in each of two control experiments), we believe that the LMTK⁻ clones transformed with CHV79 DNA are true DNA transformants and not revertants. Most of the colonies isolated were true transferants, although three B₈₂ clones were amethopterin resistant and one TG8 clone was presumably a revertant because it displayed the mouse enzyme.

Table 2. Characterization of the phenotypic marker enzyme in transferrent clones

Cells/DNA	Total colonies	Donor phenotype*	
		Present	Absent
HPRT ⁺ colonies			
A ₉ /human placenta	1	1	0
A ₉ /HeLa	1	1	0
A ₉ /CHV79	1	1	0
TG8/CHV79	22	10	1 [†]
CHTG49/HeLa	7	5 [‡]	0
CHTG49/L ₉₂₉	7	4	0
TK ⁺ colonies			
LMTK ⁻ /HeLa	73	30 [§]	0
B ₈₂ /HeLa	2	0	2 [‡]
GalK species in TK ⁺ colonies			
LMTK ⁻ /CHV79	47	1	36
LMTK ⁻ /HeLa	73	0	47 ^{**}
B ₈₂ /CHV79	6	0	3

* All extracts examined were prepared from colonies (Exps. 1-8) that originated on independent plates unless otherwise noted.

† The HPRT activity was indistinguishable from the mouse recipient enzyme and it presumably represented a revertant.

‡ This includes only four separate plates.

§ Colonies were obtained from 23 independent plates.

‡ These extracts contained no TK activity and the cells could be propagated in amethopterin-containing medium not supplemented with hypoxanthine or thymidine.

‡ These represent colonies from 29 separate plates.

** There were 35 independent plates.

Stability of the TK⁺ and HPRT⁺ Phenotypes. The stability of the TK⁺ or HPRT⁺ phenotypes in most of the transferrent clones was examined after growth in nonselective medium for intervals of 1 week, 1 month, or both. After 1 week in nonselective medium, all these tk transformants displayed some instability; after 1 month, 80% of the transferrent clones were composed of cell populations in which at least 80% of the cells could be cloned in the presence of 5-bromodeoxyuridine (BrdUrd) and presumably had lost the tk gene. Although there were insufficient data to permit precise determinations of the rate of tk loss in these clones, the rate was quite high. For example, if cells appeared that had lost the tk gene at a rate of 5 or 10% per cell generation, the cell populations would contain only 21 or 4%, respectively, TK⁺ cells after 30 generations (i.e., about 1 month). The rate of loss would frequently be underestimated, of course, due to the presence of some stable TK⁺ cells within a cell population when it was tested.

The rate of loss of the transferred *hprt* gene from A₉ cells was at least as great as that of the transferred marker from tk⁺ transferrents. The assessment of *hprt* gene stability in TG8 transferrent clones was complicated by moderately poor (i.e., 10-40%) plating efficiency of the TG8 cells in nonselective minimal essential medium. Those colonies that appeared were diffuse and difficult to count. However, about half of the TG8 transferrents also appeared to be at least moderately unstable.

Cotransfer of tk and galk. A number of studies (20-25) indicate that the tk and galk loci are closely linked in humans and rodents. It was recently estimated (24) that they are separated by no more than 0.04% of the haploid human genome. Thus, it was of interest to determine whether any of the isolated TK⁺ DNA transferrents also contained the donor species of GalK. The frequency of cotransfer could provide a basis for estimating the size of the transferred DNA fragment. Extracts of 87 TK⁺ DNA transferrents originating on 65 independent plates were analyzed by starch gel electrophoresis. The donor form of GalK was detected in a single clone, DE 4-3, a TK⁺ clone transformed with Chinese hamster DNA (Fig. 2, Table 2). The possibility of contamination of this clonal cell line with a few Chinese hamster cells was eliminated by fluorescent microscopy of over 200 cells from clone DE 4-3 stained with Hoechst 33258. All

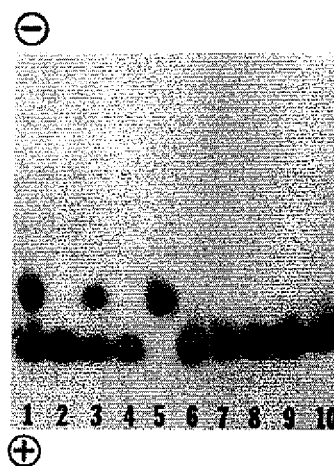


FIG. 2. Starch gel electrophoresis of mouse and Chinese hamster GalK. The extracts are: 5, CHV79; 6, L₉₂₉; 1 and 3, extracts of DE 4-3 grown from two different preparations of frozen cell stocks; 2 and 4, extracts from two other clones of TK⁺ LMTK⁻ cells resulting from transformation with CHV79 DNA; 7-10, four separate BrdUrd-resistant subclones of DE 4-3 that were isolated after growth in nonselective medium.

the cells had the chromosomal or nuclear fluorescence typical of mouse cells. To determine whether the two genes were co-transferred on a single molecule of DNA or if there was fortuitous transfer of both genes on separate DNA fragments, DE 4-3 was grown for one week in nonselective medium and then cloned in BrdUrd to select cells that had lost the *tk* gene. Four *tk*⁻ clones were isolated and expanded, and extracts from them were analyzed by starch gel electrophoresis (Fig. 2). Concordant segregation of *galk* and *tk* occurred in all four clones, which had been back-selected for loss of *tk* alone, suggesting that both genes had been transferred on a single fragment of DNA.

The detection of cotransfer of *tk* and *galk* genes was surprising and suggested that the size of the input DNA may be large. The molecular weight of single-stranded DNA was determined by velocity sedimentation in isokinetic alkaline sucrose gradients (26, 27) and ranged from 3 to 17×10^6 (i.e., 10–55 kilobases), somewhat smaller than the DNA of metaphase chromosomes lysed on alkaline gradients (28). The size of nondenatured DNA was measured by electrophoresis in a neutral 0.5% agarose gel. The modal DNA size in all preparations was at least 100 kilobase pairs, and the HeLa and Chinese hamster DNA preparations were considerably larger.

DISCUSSION

The efficient transfer of cellular genes by using purified high molecular weight DNA will be an important tool as a bioassay in the isolation of individual genes. However, our results demonstrate that the efficiency of the method, and thus its value as a bioassay, may be dependent upon the characteristics of the recipient cell. The results reported here indicate that the frequency of DNA-mediated transfer of *hprt* can be enhanced by the appropriate choice of recipient cell lines. For example, it was found that mouse A₉ cells were inefficient recipients for *hprt* transfer but that the mouse TG8 and Chinese hamster CHTG49 *hprt*⁻ cell lines were transformed by the same DNA preparations at a high rate, close to that observed for the transfer of *tk* into LMTK⁻ cells. This apparent difference in uptake and expression of genes by the recipient cells might be due to differences in the fraction of cells in any cell line that are competent to take up DNA at a given time. Wigler *et al.* (29) demonstrated, in experiments involving cotransformation of LMTK⁻ cells with physically unlinked genes, that there is a subpopulation (less than 10%) of competent cells in which transformation occurs. The factors that determine competency are not yet known, but they could include the specific stage of the cell cycle, the rate of DNA uptake, the rate of degradation of ingested DNA by lysosomal nucleases, or the level of enzymes involved in DNA repair processes.

In contrast to our results with DNA-mediated gene transfer, recipient cell specificity does not seem to be a factor in chromosome-mediated gene transfer. No significant differences have been observed in the frequency of chromosome-mediated gene transfer of *tk* into LMTK⁻ cells (21) or mouse B₃₂ recipients and *hprt* into A₉ (10, 11, 30) or CHTG49 recipients (9). Miller and Ruddle (13) have reported that the frequency of chromosome-mediated gene transfer of *tk* was enhanced by applying the chromosomes as a calcium phosphate precipitate and subsequently exposing the cells to Me₂SO. Using this same procedure for chromosome-mediated gene transfer, we have observed that the frequency for transfer of *tk* into LMTK⁻ cells is similar to the frequency of *hprt* transfer into A₉ recipient cells (unpublished results). However, a nearly identical procedure employed in the present investigation of DNA-mediated transfer resulted in markedly different transfer frequencies of *tk* into LMTK⁻ cells and *hprt* into A₉ cells. The explanation for the differences in recipient cell specificity of the two methods

is not clear. The significant observation is that the *hprt* gene, which is an important medical, biochemical, and genetic marker, can be transferred efficiently by both methods under the appropriate conditions.

The detection of cotransfer of the linked loci *tk* and *galk* was unexpected. Wigler *et al.* (29) have demonstrated that physically unlinked genes can be cotransferred with relatively high efficiency under appropriate conditions. However, this involved the use of high input ratio (i.e., 2000–20,000) of the purified nonselected gene to the selectable marker. If *tk* and *galk* had been transferred into a cell on separate DNA fragments, each fragment could have been lost independently and rapidly during subsequent propagation of the cells. The retention of a high level of the nonselected *Galk* activity during the establishment of the clonal cell line and the concordant segregation of both genes in four independent clones isolated during back-selection in BrdUrd suggests that the two genes were transferred on one fragment. More recent results greatly strengthen this interpretation. Cloned populations of cells were grown in BrdUrd and HAT for the same period of time. Both genes were maintained in all 9 clones isolated in HAT and lost in all 12 clones isolated in BrdUrd. In addition, no donor *Galk* activity could be detected in a noncloned population grown in BrdUrd. However, another interpretation of our results is that the two genes were transferred into the cell on separate DNA fragments and were then fortuitously joined. A recent report by Klobutcher and Ruddle (31) suggests that such ligation of fragments may occur in transgenes after chromosome-mediated gene transfer. They reported concordant segregation of asynthetic markers in two out of seven LMTK⁻ clones into which the HeLa *tk* had been transferred. At present we cannot determine which mechanism is responsible for the cotransfer of these markers in our experiments.

The absolute frequency of *tk* and *galk* cotransfer clearly cannot be determined from the present data, because cotransfer was detected in only one instance. This frequency is not greater than about 1–2%, but it could be at least an order of magnitude lower. Presently it is not possible to predict the frequency of cotransfer of any two single-copy genes on independent fragments because the fraction of cells that are competent for transformation with DNA is unknown (29). For example, assuming a frequency of 10^{-6} for transfer of any single marker, the cotransfer of independent markers at a frequency of 1% would be anticipated if only a 10^{-4} fraction of recipient cells are competent for DNA transformation.

The frequency of cotransfer of *tk* and *galk* by chromosome-mediated gene transfer is about 25% (21–23). Assuming that the genes were transferred on one piece of DNA, our findings indicate that the size of functional genetic fragments transferred by the DNA-mediated gene transfer is significantly smaller than that transferred by chromosome-mediated gene transfer. However, the magnitude of the size difference cannot be ascertained from the current information. In a simple model involving uptake of relatively uniform fragments resulting from random breaks in the donor genome, this size difference could obviously be quite small. For example, the frequencies of cotransfer observed by using the two techniques could be produced by DNA fragments with about the size of the *tk-galk* intergenic distance, while a 25% rate of cotransfer would occur with fragments only one-third longer. If cotransfer results from infrequent uptake of very large fragments from a rather heterogeneous population of fragment sizes, the difference in the sizes of most fragments transferred by these two procedures could be quite large. Estimates of the fragment size resulting from chromosomal transfer based on nucleic acid hybridization studies in this laboratory (unpublished results) indicate that the

X-chromosomal fragment(s) is moderately large—in the range of 0.1–0.5% of the haploid genome. By using the same procedure, the level of transferred X-chromosomal DNA was undetectable (<0.1% of the haploid genome) in two HPRT⁺ A₉ clones transformed by human DNA.

The close linkage between *tk* and *galk* genes is well established. Previous evidence (20) permitted an estimate of 0.2% of the haploid genome as the upper limit of the distance between these two genes. A more recent report (24), based on quantitative analysis of the frequency of γ -irradiation-induced segregation of the two genes in hybrid cells, indicated an intergenic distance of only 0.04% of the haploid human genome. Because the size of this haploid genome is about 3×10^6 kilobase pairs (32), the maximum distance between the two loci is 1200 kilobase pairs. This estimated intergenic distance is about an order of magnitude larger than the modal size of the DNA used here (several hundred kilobase pairs). The disparity between these two sizes suggests that the distance between *tk* and *galk*, at least in the Chinese hamster genome, may be smaller than was previously estimated.

The current study demonstrates that an important factor for high frequency DNA transfer of the *hprt* gene is the choice of recipient cells. However, the size of a gene might also be a factor in determining transfer frequency. Gene lengths vary due to intervening sequences interspersed in the coding region of the gene (33). In addition, sequences contiguous to the gene might be essential for gene expression or integration and would thus increase the size of the DNA fragment required for expression of the transferred gene. The cotransfer of *tk* and *galk* suggests that large pieces of DNA are transferred, but additional evidence is required to prove that cotransfer occurred on one piece of DNA as well as to establish any correlation between gene size and transfer frequency.

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Introduction and expression of a rabbit β -globin gene in mouse fibroblasts

(DNA-mediated gene transfer/cotransformation/intervening sequences/gene regulation)

B. WOLD*, M. WIGLER†, E. LACY‡, T. MANIATIS‡, S. SILVERSTEIN*, AND R. AXEL*

*College of Physicians and Surgeons, Columbia University, New York, New York 10032; †Cold Spring Harbor Laboratories, Cold Spring Harbor, New York 11724; and ‡Division of Biology, California Institute of Technology, Pasadena, California 91125

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ABSTRACT The cloned chromosomal rabbit β -globin gene has been introduced into mouse fibroblasts by DNA-mediated gene transfer (transformation). In this report, we examine the expression of the rabbit gene in six independent transformants that contain from 1 to 20 copies of the cloned globin gene. Rabbit globin transcripts were detected in two of these transformants at steady-state concentrations of 5 and 2 copies per cell. The globin transcripts from one cell line are polyadenylated and migrate as 9S RNA on methylmercury gels. These transcripts reflect correct processing of the two intervening sequences but lack 48 ± 5 nucleotides present at the 5' terminus of rabbit erythrocyte globin mRNA.

Cellular genes coding for selectable biochemical functions can be stably introduced into cultured mammalian cells by DNA-mediated gene transfer (transformation) (1, 2). Biochemical transformants are readily identified by the stable expression of a gene coding for a selectable marker. These transformants represent a subpopulation of competent cells that integrate other physically unlinked genes for which no selective criteria exist (3). In this manner, we have used a viral thymidine kinase (tk) gene as a selectable marker to isolate mouse cell lines that are stably transformed with the tk gene along with bacteriophage ϕ X174, plasmid pBR322, or the cloned chromosomal rabbit β -globin gene sequences (3).

Cotransformed mouse fibroblasts containing the rabbit β -globin gene provide an opportunity to study the expression and subsequent processing of these sequences in a heterologous host. In this report, we demonstrate the expression of the transformed rabbit β -globin gene generating a discrete polyadenylated 9S species of globin RNA. This RNA results from correct processing of both intervening sequences, but lacks approximately 48 nucleotides present at the 5' terminus of mature rabbit β -globin mRNA.

MATERIALS AND METHODS

Cell Culture. Murine Ltk⁻ aprt⁻ cells are adenine phosphoribosyltransferase-negative derivatives of Ltk⁻ clone 1D cells (4) that were originally isolated and characterized by R. Hughes and P. Plagemann. Cells were maintained in growth medium and prepared for transformation as described (5).

Transformation and Selection. The transformation protocol, selection for tk⁺ transformants, and maintenance of transformant cell lines were as described (5).

DNA Isolation. DNA was extracted from cultured L cells as described (5). Recombinant phage containing the rabbit β -globin gene in the λ phage vector Charon 4A were grown and

purified, and DNA was isolated as described (6). The herpes virus DNA fragment containing the tk gene was purified from total DNA of herpes simplex virus strain F (7). Intact herpes virus DNA was digested with the restriction endonuclease *Kpn* I and fractionated by agarose gel electrophoresis, and the 5.1-kilobase pair (kbp) fragment containing the tk gene was extracted from the gel as described (8).

RNA Isolation. Total RNA was isolated from logarithmic-phase cultures of transformed L cells by successive extractions with phenol at pH 5.1, phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol), and chloroform/isoamyl alcohol (24:1, vol/vol). After ethanol precipitation, the RNA was digested with DNase (9) and precipitated with ethanol. Nuclear and cytoplasmic fractions were isolated as described (5) and RNAs were extracted as described above. Cytoplasmic polyadenylated RNA was isolated by oligo(dT)-cellulose chromatography (10).

cDNA Synthesis. Rabbit and mouse cDNAs were prepared by using avian myeloblastosis virus reverse transcriptase (RNA-dependent DNA polymerase) (obtained from J. W. Beard), as described (11).

DNA Filter Hybridizations. Cellular DNA was digested with restriction endonucleases, electrophoresed on agarose slab gels, transferred to nitrocellulose filter sheets, and hybridized with ³²P-labeled DNA probes as described by Wigler *et al.* (5).

Solution Hybridizations. ³²P-Labeled globin cDNAs (specific activities of $2-9 \times 10^8$ cpm/ μ g) were hybridized with excess RNA in 0.4 M NaCl/25 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.5/5 mM EDTA at 75°C. Incubation times did not exceed 70 hr. *R*₀t's were calculated as moles of RNA nucleotides per liter times time in seconds. The fraction of cDNA rendered resistant to the single-strand nuclease S1 in hybridization was determined as described (10).

RNA Filter Hybridizations. RNA was electrophoresed through 1% agarose slab gels (17 \times 20 \times 0.4 cm) containing 5 mM methylmercury hydroxide as described by Bailey and Davidson (12). The concentration of RNA in each slot was 0.5 μ g/ μ l. Electrophoresis was at 110 V for 12 hr at room temperature.

RNA was transferred from the gel to diazotized cellulose paper as described by Alwine *et al.* (13) by using pH 4.0 citrate transfer buffer. After transfer, the RNA filter was incubated for 1 hr with transfer buffer containing carrier RNA at 500 μ g/ml. The RNA on the filters was hybridized with cloned DNA probe at 50 ng/ml labeled by ³²P nick translation (14) to specific activities of $2-8 \times 10^8$ cpm/ μ g. Reaction volumes were

Abbreviations: tk, thymidine kinase; kbp, kilobase pairs; Pipes, 1,4-piperazinediethanesulfonic acid; *R*₀t, product of RNA concentration (moles of nucleotide per liter) and incubation time (seconds).

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25 $\mu\text{l}/\text{cm}^2$ of filter. Hybridization was in 4X standard saline citrate (0.15 M NaCl/0.015 M sodium citrate)/50% formamide at 57°C for 36–48 hr.

After hybridization, filters were soaked in two changes of 2X standard saline citrate/25 mM sodium phosphate/1.5 mM sodium pyrophosphate/0.1% sodium dodecyl sulfate/5 mM EDTA at 37°C for 30 min with shaking to remove formamide. Successive washes were at 68°C with 1X and 0.1X standard saline citrate containing 5 mM EDTA and 0.1% sodium dodecyl sulfate for 30 min each.

Berk-Sharp Analysis of Rabbit β -Globin RNA in Transformed Mouse L Cells. The hybridizations were carried out in 80% (vol/vol) formamide (Eastman)/0.4 M Pipes, pH 6.5/0.1 mM EDTA/0.4 M NaCl (15, 16) for 18 hr at 51°C for the 1.8 kbp *Hha* I fragment and 49°C for the *Pst* I fragment. The hybrids were treated with S1 nuclease and analyzed by a modification of the procedure described by Berk and Sharp (16).

RESULTS

Transformation of mouse cells with the rabbit β -globin gene

We have performed cotransformation experiments with the chromosomal adult rabbit β -globin gene, using the purified herpes virus tk gene as a biochemical marker. The addition of the tk gene to mutant Ltk⁻ mouse fibroblasts results in the appearance of stable transformants that can be selected by their ability to grow in hypoxanthine/aminopterin/thymidine (HAT) medium. Cells were cotransformed with a β -globin gene clone designated R β G1, which consists of a 15.5-kbp insert of rabbit DNA carried in the bacteriophage λ cloning vector

Charon 4A (Fig. 1A) (unpublished data). The purified tk gene was mixed with a 100-fold molar excess of intact recombinant DNA from clone R β G1. This DNA was then exposed to mouse Ltk⁻ cells under transformation conditions previously described (5). After 2 weeks in selective medium, tk⁺ transformants were observed at a frequency of one colony per 10⁶ cells per 20 μg of tk gene. Clones were picked and grown into mass culture.

We then asked if the tk⁺ transformants also contain rabbit β -globin sequences. High molecular weight DNA from eight transformants was cleaved with the restriction endonuclease *Kpn* I. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and these filters were then annealed with nick-translated globin [³²P]DNA [blot hybridization (17)]. Cleavage of this recombinant phage with the enzyme *Kpn* I generates a 4.7-kbp fragment that contains the entire adult β -globin gene, along with 1.4 kbp of 5' flanking information and 2.0 kbp of 3' flanking information (Fig. 1A). This fragment was purified by gel electrophoresis and nick translated to generate a hybridization probe. Blot hybridization experiments showed that the 4.7-kbp *Kpn* I fragment containing the globin gene was present in the DNA of six of the eight tk⁺ transformants. In three of the clones (Fig. 2, lanes E, F, and H), additional rabbit globin bands were observed, which probably resulted from the loss of at least one of the *Kpn* I sites during transformation. The number of rabbit globin genes integrated in these transformants was variable: some clones contained a single copy of the gene (Fig. 2, lanes J and K), whereas others contained up to 20 copies of the heterologous gene. It should be noted that the β -globin genes of mouse and rabbit are partially homologous. However, we do not observe hybridization of the rabbit β -globin probe to *Kpn*-cleaved mouse DNA, presumably because *Kpn* cleavage of mouse DNA leaves the β -gene cluster in exceedingly high molecular weight fragments not readily detected in these experiments (Fig. 2). These results demonstrate the introduction of the cloned chromosomal rabbit β -globin gene into mouse cells by DNA-mediated gene transfer.

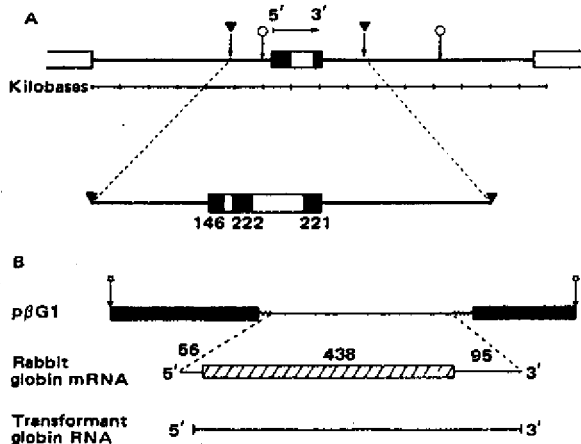


FIG. 1. (A) Structure of the rabbit β -globin genomic clone R β G1. The solid box represents the mRNA coding sequence in the adult β -globin gene. The clear regions bounded by coding sequence indicate the intervening sequences within the β -globin gene. The larger 3' intervening sequence is about 600 base pairs long and the smaller 5' sequence (shown only in the lower map) is about 125 base pairs long. Restriction sites are indicated by arrows: ∇ , *Kpn* I; \circ , *Pst* I. (B) Structure of the cDNA clone p β G1 and rabbit β -globin mRNA. The *Hha* I restriction fragment of p β G1 is shown. The heavy black lines indicate pMB9 plasmid vector sequence and the thin straight line indicates rabbit mRNA sequence: \star *Hha* I sites. The map of rabbit globin mRNA shows the 438-nucleotide translated region bounded by the 5' 56-nucleotide untranslated region and the 3' 95-nucleotide untranslated region. The bottom map is of cytoplasmic polyadenylated rabbit globin RNA from transformant cell line 6, which lacks approximately 48 nucleotides of 5' mRNA sequence (see Results).

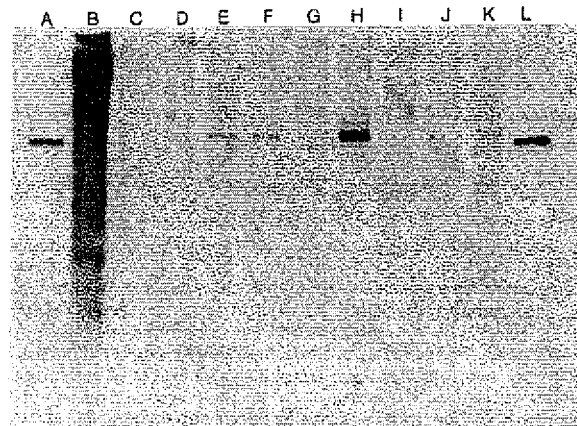


FIG. 2. Rabbit β -globin genes in transformed mouse L cells. High molecular weight DNA from eight independent cotransformant clones was digested with *Kpn* I and electrophoresed on a 0.7% agarose gel. The DNA was denatured *in situ* and transferred to nitrocellulose filters, which were then annealed with a ³²P-labeled 4.7-kbp fragment containing the rabbit β -globin gene. Lanes A and L, 50 μg of the 4.7-kbp *Kpn* I fragment of R β G1; lane B, 15 μg of rabbit liver DNA digested with *Kpn*; lane C, 15 μg of Ltk⁻ apt⁻ DNA; lanes D–K, 15 μg of DNA from each of eight independently isolated tk⁺ transformants.

Rabbit β -globin sequences are transcribed in mouse transformants

The cotransformation system we have developed may provide a functional assay for cloned eukaryotic genes if these genes are expressed in the heterologous recipient cell. Six transformed cell clones were therefore analyzed for the presence of rabbit β -globin RNA sequences. In initial experiments we performed solution hybridization reactions to determine the cellular concentration of rabbit globin transcripts in our transformants. A radioactive cDNA copy of purified rabbit α - and β -globin mRNA was annealed with a vast excess of cellular RNA. Because homology exists between the mouse and rabbit globin sequences, it was necessary to determine experimental conditions such that the rabbit globin cDNAs did not form stable hybrids with mouse globin mRNA but did react completely with homologous rabbit sequences. At 75°C in the presence of 0.4 M NaCl, over 80% hybridization was observed with the rabbit globin mRNA, whereas the heterologous reaction with purified mouse globin mRNA did not exceed 10% hybridization. The $R_{0t_{1/2}}$ of the homologous hybridization reaction was 6×10^{-4} , a value consistent with a complexity of 1250 nucleotides contributed by the α - plus β -globin sequences in our cDNA probe (10).

This rabbit globin cDNA was used as a probe in hybridization reactions with total RNA isolated from six transformed cell lines (Fig. 3 and data not shown). Total RNA from transformed clone 6 (Fig. 2, lane H) protected 44% of the rabbit cDNA at completion, the value expected if only β -gene transcripts were present. This reaction displayed pseudo-first-order kinetics with an $R_{0t_{1/2}}$ of 2×10^3 . A second transformant (Fig. 2, lane E) reacted with an $R_{0t_{1/2}}$ of 8×10^3 (data not shown). No significant hybridization was observed at $R_{0t} \geq 10^4$ with total RNA preparations from the four additional transformants.

We have characterized the RNA from clone 6 in greatest detail. RNA from this transformant was fractionated into nuclear and cytoplasmic populations to determine the intracellular localization of the rabbit globin RNA. The cytoplasmic RNA

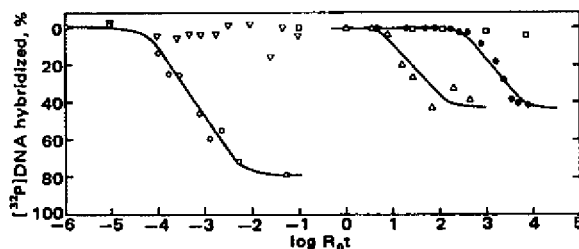


FIG. 3. Hybridization of rabbit globin cDNA with RNA from a mouse L cell transformant containing R β G1 sequences. The curves represent single pseudo-first-order kinetic components fit to the data by least-squares methods. O, Hybridization of rabbit globin [32 P]cDNA ($7-9 \times 10^6$ cpm/ μ g) with excess globin template RNA. At termination, 80% of the cDNA is in hybrid. The rate constant is $1.1 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$. ∇ , Hybridization of rabbit globin cDNA with mouse globin mRNA. Δ , Hybridization of excess polyadenylated cytoplasmic RNA from transformant 6 (see text) with rabbit globin cDNA. The rate constant is $2.8 \times 10^{-2} \text{ M}^{-1} \text{ sec}^{-1}$. The extent of reaction was 43% after normalization for the 70% reactivity of the cDNA at the time of this measurement. \bullet , Hybridization of excess total cellular RNA from transformant 6 with rabbit globin cDNA. At termination, 43% of the [32 P]cDNA was in hybrid. The rate constant is $13.5 \times 10^{-4} \text{ M}^{-1} \text{ sec}^{-1}$. \square , Hybridization of excess nuclear RNA from transformant 6 with rabbit globin cDNA. The S1 resistance of cDNA at zero time has been subtracted from all hybridization values. These background values were 5% and 14% for the cDNA preparations used in this experiment.

was further fractionated by oligo(dT)-cellulose chromatography into poly(A) $^{+}$ and poly(A) $^{-}$ RNA. Poly(A) $^{+}$ cytoplasmic RNA from clone 6 hybridizes with the rabbit cDNA with an $R_{0t_{1/2}}$ of 25. This value is 1/80th the $R_{0t_{1/2}}$ observed with total cellular RNA, consistent with the observation that poly(A) $^{+}$ cytoplasmic RNA is 1-2% of the total RNA in a mouse cell. Hybridization is not detectable with either nuclear RNA or cytoplasmic poly(A) $^{-}$ RNA at R_{0t} values of 1×10^4 and 2×10^4 , respectively. The steady-state concentration of rabbit β -globin RNA present in our transformant can be calculated from the $R_{0t_{1/2}}$ to be about five copies per cell, with greater than 90% localized in the cytoplasm.

Several independent experiments argue that the globin RNA detected derives from transcription of the rabbit DNA sequences present in this transformant: (i) cDNA was prepared from purified 9S mouse globin RNA. This cDNA does not hybridize with poly(A) $^{+}$ RNA from clone 6 at R_{0t} values at which the reaction with rabbit globin cDNA is complete (Fig. 3). (ii) Rabbit globin cDNA does not hybridize with total cellular RNA obtained with tk $^{+}$ globin $^{-}$ transformants at R_{0t} values exceeding 10^4 . (iii) The hybridization we observe does not result from duplex formation with rabbit globin DNA possibly contaminating the RNA preparations. Rabbit cDNA was annealed with total cellular RNA from clone 6, the reaction product was treated with S1 nuclease, and the duplex was subjected to equilibrium density centrifugation in cesium sulfate under conditions that separate DNA-RNA hybrids from duplex DNA. The S1-resistant cDNA banded at a density of 1.54 g/ml, as expected for DNA-RNA hybrid structures (data not shown). These data, along with the observation that globin RNA is polyadenylated, demonstrate that the hybridization we observe with RNA preparations does not result from contaminating DNA sequences.

Characterization of rabbit globin transcripts in transformed cells

In rabbit erythroblast nuclei, the β -globin gene sequences are detected as a 14S precursor RNA that reflects transcription of two intervening sequences that are subsequently removed from this molecule to generate a 9S messenger RNA (unpublished results). It was therefore of interest to determine whether the globin transcripts we detected exist as a discrete 9S species, which is likely to reflect appropriate splicing of the rabbit gene transcript by the mouse fibroblast. Cytoplasmic poly(A)-containing RNA from clone 6 was electrophoresed on a methylmercury/agarose gel (12) and transferred to diazotized cellulose paper (13, 18). After transfer, the RNA on the filters was hybridized with DNA from the plasmid p β G1, which contains rabbit β -globin cDNA sequences (19). Using this 32 P-labeled probe, we observed a discrete 9S species of RNA in the cytoplasm of the transformant, which comigrated with rabbit globin mRNA isolated from rabbit erythroblasts (Fig. 4). Hybridization to 9S RNA species was not observed in parallel lanes containing either purified mouse 9S globin RNA or poly(A)-containing cytoplasmic RNA from a tk $^{+}$ transformant containing no rabbit globin genes.

We were unable in these experiments to detect the presence of a 14S precursor in nuclear RNA populations from the transformants. This is not surprising, because the levels expected in nuclear RNA, given the observed cytoplasmic concentration, are likely to be below the limits of detection for this technique. The 5' and 3' boundaries of the rabbit globin sequences expressed in transformed fibroblasts along with the internal processing sites can be defined more accurately by hybridizing this RNA with cloned DNAs, followed by S1 nuclease digestion and subsequent gel analysis of the DNA products (16). When

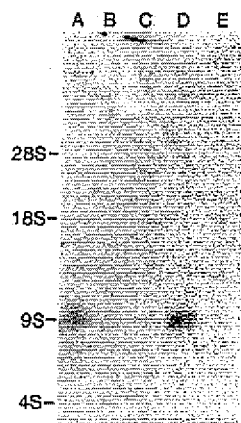


FIG. 4. Sizing of cytoplasmic polyadenylated rabbit globin transcripts from transformant 6. RNA was electrophoresed in a 1% methylmercury/agarose gel and the RNA was transferred to diazotized cellulose paper. The positions of 28S, 18S, and 4S RNAs on the gel were determined optically after staining with ethidium bromide. The RNA on the filter was hybridized with ^{32}P -labeled plasmid DNA (p βG1) containing the rabbit β -globin cDNA sequence. Lane A, 1 ng of purified 9S polyadenylated RNA from rabbit reticulocytes, plus 25 μg of carrier chicken oviduct RNA. Lane B, 50 μg of purified 9S polyadenylated RNA from rabbit reticulocytes, plus 25 μg of carrier chicken oviduct RNA. Lane C, 1 ng of purified 9S polyadenylated RNA from mouse reticulocytes plus 25 μg of carrier RNA. Lane D, 30 μg of polyadenylated cytoplasmic RNA from transformant 6. Lane E, 30 μg of cytoplasmic polyadenylated RNA from a transformant containing no rabbit globin genes.

β -globin mRNA from rabbit erythroid cells was hybridized with cDNA clone p βG1 (Fig. 1B) under appropriate conditions, the entire 576-base pair insert of cDNA was protected from S1 nuclease attack. When this cDNA clone was hybridized with RNA from our transformant, surprisingly, a discrete DNA band was observed at 525 base pairs, but not at 576 base pairs (Fig. 5). These results suggest that, in this transformant, rabbit globin RNA molecules are present that have a deletion in a portion of the globin mRNA sequence at the 5' or 3' termini. To distinguish between these possibilities, DNA of the λ clone, R βG1 , containing the chromosomal rabbit β -globin sequence hybridized with transformed fibroblast RNA. The hybrid formed was treated with S1 nuclease, and the protected DNA fragments were analyzed by alkaline agarose gel electrophoresis and identified by Southern blotting procedures (17). Because the rabbit β -globin gene is interrupted by two intervening sequences, the hybridization of mature rabbit mRNA to R βG1 DNA generates three DNA fragments in this sort of analysis: a 146-base pair fragment spanning the 5' terminus to the junction of the small intervening sequence, a 222-base pair internal fragment bridging the small and large intervening sequences, and a 221-base pair fragment spanning the 3' junction of the large intervening sequence to the 3' terminus of the mRNA molecule (Fig. 1A). When transformant RNA was analyzed in this fashion, we observed a 222-base pair fragment and an aberrant fragment of 100 base pairs but no 146-base pair fragment (Fig. 5). Hybridization with a specific 5' probe showed that the internal 222 base pair fragment was present (data not shown). The sum of the protected lengths equaled the length of the DNA fragment protected by using the cDNA clone. Taken together, these results indicate that although the intervening sequences expressed in transformed mouse fibroblast are removed from the RNA transcripts precisely, the 5' termini of the cytoplasmic transcripts we observe do not contain

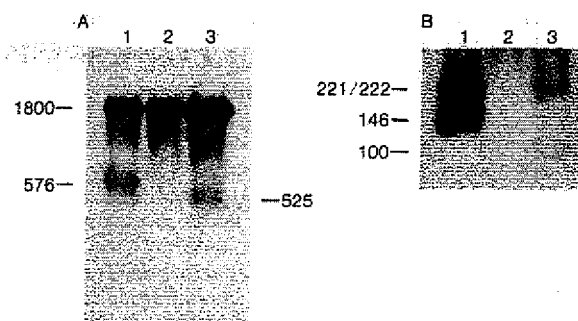


FIG. 5. Characterization of rabbit β -globin RNA in transformed mouse L cells. Numbers of base pairs are given beside the autoradiograms. (A) Both total rabbit reticulocyte RNA and poly(A) $^{+}$ RNA purified from cell line 6 were hybridized to the 1.8-kbp *Hha* I fragment from plasmid p βG1 (Fig. 1B) and analyzed as described by Berk and Sharp (16). Lane 1, 0.2 μg of total reticulocyte RNA was hybridized to 20 ng of the 1.8-kbp *Hha* I fragment in 5 μl . Lane 2, 18 ng of the 1.8-kbp *Hha* I fragment was hybridized in 2.5 μl in the absence of any added RNA. Lane 3, 30 μg of poly(A) $^{+}$ RNA purified from cell line 6 was hybridized to 75 ng of the 1.8-kbp *Hha* I fragment in 10 μl . The 1800-base pair band is the renatured *Hha* fragment. (B) Both total rabbit reticulocyte RNA and poly(A) $^{+}$ RNA purified from cell line 6 were hybridized to a 5.60-kbp *Pst* I fragment containing the genomic copy of the rabbit β -globin gene. The Berk-Sharp analysis was carried out by a procedure to be described elsewhere. Only the bottom half of the autoradiogram is shown and therefore lane-specific background present in lanes 1 and 3, as well as in the RNA $^{-}$ control (lane 2) is not shown. We believe that this background results from the formation of DNA-DNA duplexes between a small number of nicked *Pst* fragments prior to S1 treatment. Lane 1, 0.35 μg of total rabbit reticulocyte RNA was hybridized to 0.1 μg of the 5.60-kbp *Pst* I fragment in 10 μl . Lane 2, 0.12 μg of the *Pst* I fragment was hybridized in 10 μl in the absence of any RNA. Lane 3, 30 μg of poly(A) $^{+}$ RNA purified from cell line 6 was hybridized to 0.12 μg of the 5.60-kbp *Pst* I fragment in 10 μl .

about 48 ± 5 nucleotides present in mature 9S RNA of rabbit erythroblasts.

DISCUSSION

In these studies, we have constructed mouse cell lines that contain the rabbit β -globin gene and have analyzed the ability of the mouse fibroblast recipient to transcribe and process this heterologous gene. Solution hybridization experiments in concert with RNA blotting techniques indicate that, in at least one transformed cell line, rabbit globin sequences are expressed in the cytoplasm as a polyadenylated 9S species. Correct processing of the rabbit β -globin gene has also been observed in tk $^{+}$ mouse cell transformants in which the globin and tk plasmids have been ligated prior to transformation (20). Similar results have been obtained by using a viral vector to introduce the rabbit globin gene into monkey cells (21, 22). Taken together, these results suggest that nonerythroid cells from heterologous species contain the enzymes necessary to correctly process the intervening sequences of a rabbit gene whose expression usually is restricted to erythroid cells.

The level of expression of rabbit globin sequences in our transformant is low: five copies of globin RNA are present in the cytoplasm of each cell. Our results indicate that the two intervening sequences present in the original globin transcript are processed and removed at loci indistinguishable from those observed in rabbit erythroid cells. Surprisingly, 45 nucleotides present at the 5' terminus of mature rabbit mRNA are absent

from the β -globin RNA sequence detected in the cytoplasm of the transformant we have examined. It is possible that incorrect initiation of transcription occurs about the globin gene in this mouse cell line. Alternatively, the globin sequences we detect may result from transcription of a long precursor that ultimately must undergo 5' processing to generate the mature 9S species. Incorrect processing at the 5' terminus in the mouse fibroblast could be responsible for our results. At present, it is difficult to distinguish among these alternatives. Because we are restricted in our analysis to a single transformant, we do not know whether these observations are common to all transformants expressing the globin gene or reflect a rare but interesting aberration. It should be noted, however, that in similar experiments by Weissmann and his colleagues (20) at least a portion of the rabbit globin RNA molecules transcribed in transformed mouse fibroblasts retain the correct 5' terminus.

Several alternative explanations can be offered for the expression of globin sequences in transformed fibroblasts. It is possible that constitutive synthesis of globin RNA occurs in cultured fibroblasts (23) at levels five to six orders of magnitude below the level observed in erythroblasts. The introduction of 20 additional globin DNA templates may simply increase this constitutive transcription to the levels observed in our transformant. Alternatively, it is possible that the homologous globin gene is repressed by factors that are partially overcome by a gene dosage effect provided by the introduction of 20 additional globin genes. Finally, normal repression of the globin gene in a fibroblast may depend upon the position of these sequences in the chromosome. At least some of the newly introduced genes are likely to reside at loci distant from the resident mouse globin genes. Some of these ectopic sites may support low level transcription. Our data do not permit us to distinguish among these and other alternatives.

Although the number of rabbit globin genes within a given transformant remains stable for over a hundred generations of culture in hypoxanthine/aminopterin/thymidine (unpublished studies), it has not been possible to prove that these sequences are covalently integrated into recipient cell DNA. In previous studies, however, we have demonstrated that cotransformation of either ϕ X174 or plasmid pBR322 results in the stable integration of these sequences into high molecular nuclear DNA. In the present study, the globin gene represents a small internal segment of the high molecular weight concatenated phage DNA used in the transformation (Fig. 1A). Analysis of integration sites covalently linked to donor DNA is therefore difficult. Preliminary studies using radioactive λ sequences as a probe in DNA blotting experiments indicate that, in some of our cell lines, we have introduced a contiguous stretch of recombinant phage DNA with a minimum length of 50 kbp.

The presence of 9S globin RNA in the cytoplasm of transformants suggests that this RNA may be translated to give rabbit β -globin polypeptide. Attempts to detect this protein in cell lysates using a purified anti-rabbit β -globin antibody (kindly provided by S. Boyer) have thus far been unsuccessful. It is possible that the globin RNAs in our transformant are not translated or are translated with very low efficiency due to the absence of a functional ribosomal binding site. The cytoplasmic globin transcripts in our transformant lack about 48 nucleotides of untranslated 5' sequence (Fig. 1B), which includes 13 nu-

cleotides known to interact with the 40S ribosomal subunit in nuclease protection studies (24, 25). Even if translation did occur with normal efficiency, it is probable that the protein would exist at levels below the limits of detection of our immunologic assay due to the low level of globin RNA, and the observation that the half-life of β globin in the absence of heme and α globin may be less than 30 min (22).

These studies indicate the potential value of cotransformation systems in the analysis of eukaryotic gene expression. The introduction of wild-type genes along with native and *in vitro*-constructed mutant genes into cultured cells provides an assay for the functional significance of sequence organization. It is obvious from these studies that this analysis will be facilitated by the ability to extend the generality of cotransformation to recipient cell lines, such as murine erythroleukemia cells, that provide a more appropriate environment for the study of heterologous globin gene expression.

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Genetics

Transformation of mammalian cells with an amplifiable dominant-acting gene

(animal cell vectors/methotrexate resistance/gene amplification)

M. WIGLER*, M. PERUCHO*, D. KURTZ*, S. DANA†, A. PELLICER†, R. AXEL†, AND S. SILVERSTEIN†

*Cold Spring Harbor Laboratories, P.O. Box 100, Cold Spring Harbor, New York 11724; and †Institute of Cancer Research, Columbia University, 701 West 168th Street, New York, New York 10032

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ABSTRACT We have transferred a mutant hamster gene coding for an altered dihydrofolate reductase to wild-type cultured mouse cells by using total genomic DNA from methotrexate-resistant Chinese hamster ovary A29 cells as donor. By demonstrating the presence of hamster gene sequences in transformants we have provided direct evidence for gene transfer. Transformants selected for increased resistance to methotrexate contain increased amounts of the newly transferred gene. We have used this mutant *dhfr* gene to introduce the *Escherichia coli* antibiotic resistance plasmid pBR322 into animal cells. Amplification of the *dhfr* sequences results in amplification of the pBR322 sequences as well. The use of this gene may allow the introduction and amplification of virtually any genetic element in various new cellular environments.

The ability to transfer purified genes into cultured cells provides a unique opportunity to study the function and physical state of exogenous genes in new cellular environments. The development of systems for DNA transfer in animal cells originated with the lytic transfection of cells by using purified viral DNA (1, 2) and progressed to the stable transfer of viral transforming functions to appropriate recipient cells (3). Subsequently, viral genes from the herpesviruses coding for the biochemically selectable marker thymidine kinase (TK) (4-6) were transferred to enzyme-deficient mutant cells. Restriction fragments of herpes simplex virus type 1 encoding TK were isolated (6) and subsequently cloned into bacterial plasmids (7). Through the use of this selectable marker, virtually any gene can now be introduced into recipient cells (8, 9); however, these cells must be *tk*⁻ mutants. Other potential selection systems are available, and several laboratories have recently demonstrated the DNA-mediated transfer of cellular genes coding for selectable markers such as TK (10), adenine phosphoribosyltransferase (11) and hypoxanthine phosphoribosyltransferase (12, 13).

Dominant mutant cellular genes coding for drug resistance in principle could serve as generalized biochemical vectors for wild-type cells. Cultured mammalian cells are exquisitely sensitive to the folate antagonist methotrexate (Mtx). Mtx-resistant cell lines have been identified in three categories: (i) cells with decreased uptake of this drug (14, 15); (ii) cells that produce inordinately high levels of dihydrofolate reductase (DHFR) (16, 17); and (iii) cells with structural mutations which lower the affinity of DHFR for Mtx (18). When they were examined, cells producing high levels of DHFR were found to contain increased copy numbers of the *dhfr* gene (gene amplification) (19). An interesting Mtx-resistant variant cell line (A29) has been identified that synthesizes increased amounts of a mutant DHFR with decreased affinity for Mtx (18). We have used genomic DNA from this cell line to transfer the

mutant *dhfr* gene to wild-type Mtx-sensitive cells. Exposure of Mtx-resistant transformed cells to increasing levels of Mtx selects for cells that have amplified the transferred gene.

MATERIALS AND METHODS

Cell Culture. Mouse Ltk⁻ aprt⁻ cells (11) and NIH 3T3 cells (20) (the latter generously provided by R. A. Weinberg) were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum and antibiotics (growth medium). Chinese hamster ovary (CHO) cells and A29 cells (18), Mtx-resistant CHO derivatives (generously provided by L. Siminovitch), were maintained in growth medium supplemented with 3 times the usual concentration of nonessential amino acids. A29 cells were grown in the presence of Mtx at 20 µg/ml.

Extraction, Restriction Endonuclease Digestion, and Ligation of DNA. High molecular weight DNA was extracted from cultured cells as described (9). DNA was analyzed by electrophoresis in 0.8% agarose gels with restriction fragments of herpes simplex virus DNA as markers. Only DNA whose molecular weight average was 35×10^6 or greater was used for transformation experiments. CsCl/ethidium bromide-purified form I DNA of the *Escherichia coli* plasmid pBR322 was isolated from cultures of *E. coli* strain HB101 (21). A29 DNA and pBR322 were mixed at 3:1 mass ratios, completely digested with restriction endonuclease *Sal* I (under conditions recommended by supplier, Bethesda Research Labs), extracted once with aqueous buffer-saturated phenol/chloroform/isoamyl alcohol 25:24:1 (vol/vol), and once with chloroform/isoamyl alcohol, 24:1, and precipitated with ethanol. DNA was resuspended and ligated with T4 DNA ligase (Bethesda Research Laboratories, Rockville, MD) at 100 µg of DNA and 3 units of ligase per ml at 4°C for 24 hr in the buffers recommended by the supplier. The ligation product was reextracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol.

Transformation and Selection. Ltk⁻ aprt⁻ cells and NIH 3T3 cells were transformed with genomic DNA by the calcium phosphate coprecipitation method (2) as described (11). All DNAs were sterilized by ethanol precipitation and resuspended in 1 mM Tris-HCl/1 mM EDTA, pH 7.9. For *tk*⁺ transformation, cells were exposed to hypoxanthine/aminopterin/thymidine selective medium as described (10). Transformants resistant to Mtx were selected in growth medium containing either 0.1 or 0.2 µg of Mtx per ml with the same feeding schedule as for *tk* selection. After 2-3 weeks, colonies were isolated from individual dishes with cloning cylinders to ensure that each transformant arose from an independent event. In transformation with ligated DNAs, no more than 1 µg of pBR322 DNA was added to 10^6 cells per dish because higher

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Abbreviations: DHFR, dihydrofolate reductase; Mtx, methotrexate (amethopterin); TK, thymidine kinase; CHO, Chinese hamster ovary; kb, kilobase.

concentrations of pBR322 inhibited transformation. Ltk⁻ aprt⁻ DNA was added as carrier in these cases to a final DNA concentration of 20 µg per dish.

Filter Hybridization. DNA from parental and transformed cells was isolated, digested with restriction endonucleases, electrophoresed in 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized as described (9). The probes for these experiments were ³²P-labeled nick translated pBR322 or pdhfr-21, a cloned cDNA copy of mouse DHFR mRNA (22) (kindly provided by R. J. Kaufman and R. T. Schimke).

RESULTS

Transfer of the Mutant Hamster Dihydrofolate Reductase Gene to Mouse Cells. High molecular weight cellular DNA was prepared from wild-type Mtx-sensitive CHO cells and from A29 cells, and the ability of these DNA preparations to transfer either the *dhfr* gene or the *tk* gene to tk⁻ mouse cells (Ltk⁻ aprt⁻) or NIH 3T3 cells was tested (Table 1). DNAs from both mutant A29 and wild-type CHO cells were competent in transferring the *tk* gene to Ltk⁻ aprt⁻ cells. Mtx-resistant Ltk⁻ aprt⁻ colonies were observed after treatment of cells with DNA from A29. No colonies were observed in cells treated with wild-type CHO DNA. Similarly, there were 40-fold more Mtx-resistant 3T3 colonies after treatment of cells with A29 DNA than after treatment with wild-type CHO DNA. These data suggest that treatment of Mtx-sensitive cells with A29 DNA resulted in the transfer and expression of a mutant *dhfr* gene, thus rendering these cells insensitive to increased levels of Mtx.

In order to test this hypothesis directly, we demonstrated the presence of the hamster *dhfr* gene in DNA from transformants by using the filter hybridization method of Southern (23). A mouse *dhfr* cDNA clone (pdhfr-21) (22) that shares homology with the hamster *dhfr* gene was used as probe in these experiments. DNAs from A29, from transformants, and from *dhfr*-amplified mouse cells were cleaved with *Hind*III, electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose filters. These filters were hybridized with high-specific activity ³²P-labeled nick translated pdhfr-21 and subjected to autoradiography. This procedure visualizes restriction fragments of genomic DNA homologous to the *dhfr* probe. Prominent bands were observed at 15, 3.5, and 3 kilobases (kb) for *dhfr*-amplified mouse DNA and at 17, 7.9, 3.7, and 1.4 kb for *dhfr*-amplified hamster DNA (Fig. 1). The restriction profiles of these two species were sufficiently different to permit us to detect the hamster gene in the presence of an endogenous mouse gene.

Four Ltk⁻ aprt⁻ cell transformants resistant to Mtx were examined in this way (Fig. 1). In each transformed cell line, we observed the expected profile of bands resulting from cleavage of the endogenous mouse *dhfr* gene although at the decreased

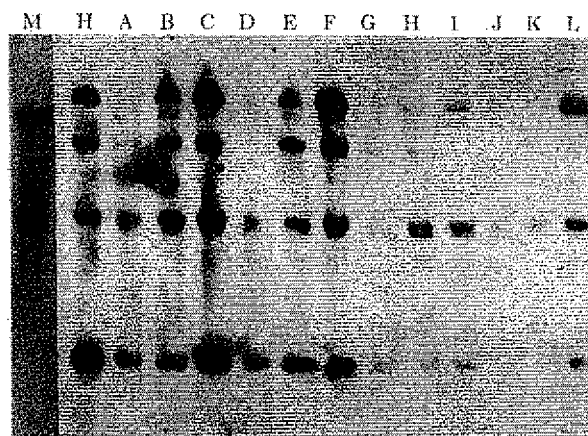


FIG. 1. Chinese hamster *dhfr* sequences are present in mouse cells. Mouse Ltk⁻ aprt⁻ cells were transformed to Mtx resistance and their DNAs were examined for the presence of CHO sequences by molecular hybridization to ³²P-labeled pdhfr-21 DNA. The hybridization profiles of 20 µg of *Hind*III-cleaved DNA from *dhfr*-amplified mouse (lane M) and the *dhfr*-amplified CHO line A29 (lane H) are shown along with the *Hind*III patterns from four Mtx-resistant cell lines derived after transformation and selection at 0.1 µg of Mtx per ml (lanes A, D, G, and J). Each of these cell lines was also grown at Mtx levels of 1 µg/ml (lanes B, E, H, and K) and 10 µg/ml (lanes C, F, I, and L) and scored for amplification of *dhfr* sequences.

intensity of unamplified *dhfr*. A series of additional bands were observed whose molecular weights were identical to those of restriction endonuclease-cleaved hamster *dhfr* gene. The 17.9-, 7.9-, and 1.4-kb bands observed in hamster DNA are diagnostic for the presence of the hamster *dhfr* gene and were present in all transformants although in disproportionate intensities. In similar studies of NIH 3T3 Mtx-resistant transformants, 12 of 12 contained bands diagnostic of hamster sequences (data not shown).

In initial experiments, we chose the lowest concentration of Mtx (0.1–0.2 µg/ml) that would decrease survival of the mouse cells to <10⁻⁷. Previous studies (18) suggested that the presence of a single mutant *dhfr* gene can render cells resistant to this concentration of Mtx. Comparison of the band intensities of the hamster *dhfr* gene fragments of transformed cell DNA with A29 suggest that our transformants contain fewer Mtx-resistant hamster genes than do donor A29 cells.

Amplification of the Transferred *dhfr* Gene. Initial Ltk⁻ aprt⁻ transformants were selected for resistance to relatively low levels of Mtx (0.1 µg/ml). For each transformant, however, it was possible to select cells resistant to increased levels of Mtx by exposing mass cultures to successively increasing concentrations of this drug. In this manner, we isolated cultures resistant to 40 µg of Mtx per ml, starting from clones that were initially resistant to 0.1 µg/ml. We next determined if increased resistance to Mtx in these transformants was associated with amplification of a *dhfr* gene and, if so, whether the endogenous mouse or the newly transferred hamster gene was amplified. DNAs from four independent transformants and their highly resistant derivatives were examined by blot hybridization. In each instance, enhanced resistance to Mtx was accompanied by an apparent increase in the copy number of the hamster gene. This is most readily seen by comparing the intensities of the 1.4-kb band (Fig. 1). In no instance did we detect amplification of the endogenous mouse *dhfr* gene. Lastly, different lines selected at equivalent Mtx concentrations appeared to contain different amounts of the *dhfr* gene.

Table 1. Transformation data

DNA source (CHO cells)	Mtx-resistant	
	colonies, no./total no. dishes	tk ⁺ colonies, no./total no. dishes
A29*	56/5† 161/5‡	25/5†
Wild type	0/5† 4/5‡	30/5†

Twenty micrograms of DNA was used to transform 10⁶ cells per dish. Mtx concentration was 0.2 µg/ml.

* CHO Pro⁻ mtx^R (18).

† Ltk⁻ aprt⁻ cells were used as recipients.

‡ NIH 3T3 cells were used as recipients.

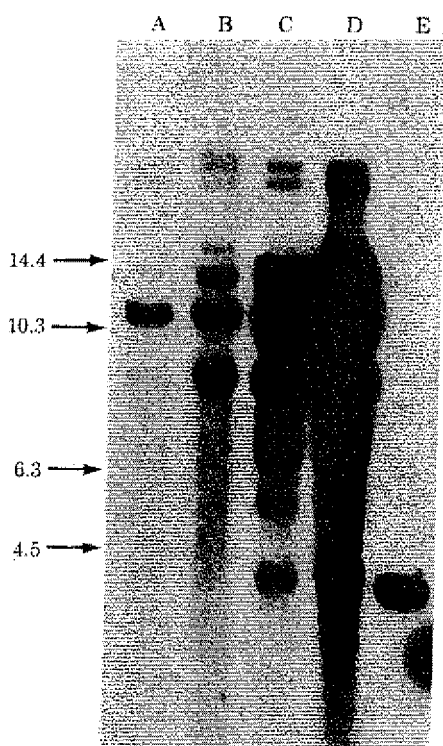


FIG. 2. Presence and amplification of pBR322 sequences in cells transformed with A29-pBR322 ligates. Cells were transformed with the ligation product of *Sal* I-cleaved A29 and pBR322 DNAs. Transformants were selected initially for resistance to 0.1 µg of Mtx per ml. After cloning, cultures were exposed to increasing concentrations of Mtx, and DNA was extracted, cleaved with *Xba* I, and analyzed for the presence of pBR322 sequences by filter hybridization. Lanes A-D, 10 µg of DNA from the SS-6 line grown in 0.1, 2, 10, or 40 µg of Mtx per ml, respectively. Lane E, 50 pg of *Pst* I-cleaved pBR322 DNA.

The *dhfr* Gene as a Generalized Transformation Vector. Selectable genes can be used as vectors for the introduction of other genetic elements into cultured cells. In previous studies, we have demonstrated that cells transformed with the *tk* gene are likely to incorporate other unlinked genes (9). The generality of this approach was tested for the selectable marker, the mutant *dhfr* gene. Total cellular DNA (20 µg) from A29 was mixed with 1 µg of *Hind*III-linearized pBR322 DNA. Recipient cells (*Ltk*⁻ *aprt*⁻) were exposed to this DNA mixture and, after 2 weeks, Mtx-resistant colonies were picked. Genomic DNA from transformants was isolated, cleaved with *Hind*III, and analyzed for the presence of pBR322 sequences. Two independent transformants were examined in this way, and multiple copies of pBR322 sequences were present in both cases (data not shown).

An alternate approach to generalized transformation involves ligation of a nonselectable DNA sequence to a selectable gene. Because the mutant *dhfr* gene is a dominant-acting gene conferring drug resistance, it can be used as a vector. Furthermore, it may be possible to amplify any genetic element ligated to this vector by selecting cells resistant to increased levels of Mtx. To explore this possibility, restriction endonucleases that do not destroy the *dhfr* gene of A29 were identified by transformation assay. One such restriction endonuclease, *Sal* I, does not destroy the transformation potential of A29 DNA. *Sal* I-cleaved A29 DNA was therefore ligated to *Sal* I-linearized pBR322. This ligation product was subsequently used in transformation experiments with *Ltk*⁻ *aprt*⁻ cells. Mtx-resistant colonies were

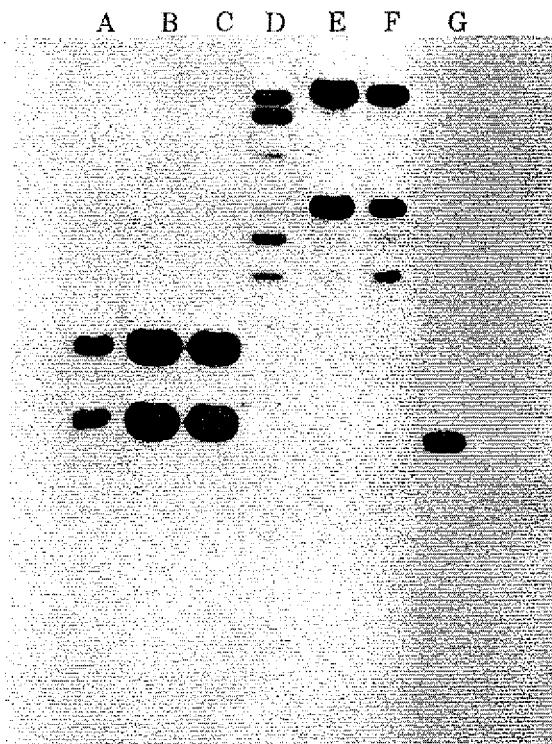


FIG. 3. As in Fig. 2. Lanes A, B, and C, 10 µg of DNA from cell line SS-1 grown in 0.1, 2, and 40 µg of Mtx per ml, respectively. Lanes D-F, 10 µg of DNA from the clone HH-1 grown in 0.1, 2, and 40 µg of Mtx per ml. Lane G, 50 pg of *Pst* I-cleaved pBR322 DNA.

picked and grown into mass culture in the presence of 0.1 µg of Mtx per ml. Mass cultures were subsequently exposed to increasing concentrations of Mtx.

DNAs were obtained from mass cultures resistant to 0.1, 2, 10, and 40 µg of Mtx per ml and the copy number of pBR322 and *dhfr* sequences was determined by blot hybridization. Six independent transformed lines were examined in this fashion. Five of these lines exhibited multiple bands homologous to pBR322 sequences. In four of these transformed clones, at least one of the pBR322-specific bands increased in intensity upon amplification of *dhfr* (Figs. 2 and 3). All pBR322 bands present in transformant SS-6 at 0.1 µg/ml continued to increase in intensity as cells were selected first at 2 µg/ml and then at 40 µg/ml (Fig. 2). We estimate that there was at least a 50-fold increase in copy number for pBR322 sequences in this cell line. In SS-1 (Fig. 3, lanes A, B, and C), two pBR322-specific bands were observed in DNA from cells resistant to 0.1 µg of Mtx per ml. These bands increased severalfold in intensity in cells resistant to 2 µg/ml. No further increase in intensity was observed, however, in cells selected for resistance to 40 µg/ml. In a third cell line, HH-1 (Fig. 3, lanes D, E, and F), two pBR322-specific bands increased in intensity upon amplification, whereas others remained constant or decreased in intensity. Thus, the pattern of amplification of pBR322 sequences we observed in these cells was quite varied. Nevertheless, the mutant *dhfr* gene can be used to introduce and subsequently amplify unselected DNA sequences in cultured animal cells.

DISCUSSION

The potential usefulness of DNA-mediated transformation in the study of eukaryotic gene expression depends to a large extent on its generality. Cellular genes coding for selectable biochemical functions have previously been introduced into mutant cultured cells (10-13). In the present study, we have

transferred a dominant-acting Mtx-resistant *dhfr* gene to wild-type cultured cells. In initial experiments, DNA from A29 cells, a Mtx-resistant CHO derivative synthesizing a mutant *dhfr*, was added to cultures of mouse Ltk⁻aprt⁻ cells or NIH 3T3 cells. Mtx-resistant colonies appeared at a frequency of about 10 colonies per 5×10^5 cells per 20 μ g of cellular DNA. Upon transformation, fewer colonies were observed with NIH 3T3 and none with Ltk⁻ cells when DNA obtained from wild-type Mtx-sensitive cells was used, although this DNA was a competent donor of the *tk* gene. The Mtx-resistant NIH 3T3 colonies obtained by using wild-type DNA as donor were not significantly above the spontaneous level of resistance (data not shown), and these colonies were not studied further. Definitive evidence that we effected transfer of a mutant hamster *dhfr* gene was the presence of the hamster gene in mouse transformants in blot hybridization experiments. In all transformants examined, we observe two sets of restriction fragments homologous to a mouse *dhfr* cDNA clone: a series of bands characteristic of the endogenous mouse gene and a second series characteristic of the donor hamster gene.

The number of copies of *dhfr* we observed in our initial transformants is low. This observation is consistent with previous studies suggesting that a single mutant *dhfr* gene is capable of rendering cells Mtx-resistant under our selective criterion (0.1 μ g of Mtx per ml) (18). Exposure of these initial Mtx-resistant transformants to stepwise increases in drug concentration results in the selection of cells with enhanced Mtx resistance. Blot analysis indicates that these cells have increased amounts of the newly transferred mutant hamster *dhfr* gene. In no transformants have we observed amplification of the endogenous mouse gene in response to selective pressure. It is likely that a single mutant gene affords significantly greater resistance to a given concentration of Mtx than does a single wild-type gene. If the frequency of amplification is low, we are merely selecting resistant variants arising from the minimal number of amplification events. It is also possible that newly transferred genes undergo amplification more readily than do endogenous genes.

We have explored the use of the mutant *dhfr* gene as a vector for the introduction and amplification of nonselectable genetic elements into cultured cells. Genomic DNA from A29 cells was cleaved with restriction enzymes and ligated to restriction endonuclease-cleaved pBR322 sequences prior to transformation. Most resulting transformants contained multiple pBR322 sequences. In many cases, amplification of *dhfr* genes resulted in the concomitant amplification of pBR322. The patterns of amplification differed among cell lines. In one transformant, all pBR322 sequences amplified with increasing Mtx concentrations. In other transformants, only a subset of the sequences amplified. In yet other lines, sequences appeared to be lost or rearranged. In some lines, amplification proceeded apace with increasing Mtx concentrations up to 40 μ g/ml whereas, in others, amplification ceased at 2 μ g/ml. It appears that the mechanisms of amplification may be quite varied. Whatever mechanisms are responsible for these complex events, it is clear that the *dhfr* amplification unit extends beyond the limits of the *dhfr* gene itself and this can be exploited to control the dosage of any gene introduced into cultured cells.

Although we have succeeded in transferring Mtx resistance to Ltk⁻aprt⁻ cells and mouse NIH 3T3 cells, we have not as yet had success with various other cell lines. In these instances either the cells were poor recipients for DNA-mediated transformation or they were already substantially resistant to Mtx. We expect that cloning a Mtx-resistant *dhfr* gene will overcome these difficulties and extend the use of this gene as amplifiable vector for wild-type cells.

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DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells

(biochemical transformation/unique genes/mutant cells)

MICHAEL WIGLER^{*†}, ANGEL PELLICER^{*}, SAUL SILVERSTEIN^{‡§}, RICHARD AXEL^{*¶}, GAIL URLAUB^{||}, AND LAWRENCE CHASIN^{||}

^{*}Institute of Cancer Research, [‡]Department of Microbiology, and [¶]Departments of Pathology and Biochemistry, Columbia University, 701 West 168th Street, New York, New York 10032; and ^{||}Department of Biological Sciences, Columbia University, New York, New York 10027

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ABSTRACT In this report, we demonstrate the feasibility of transforming mouse cells deficient in adenine phosphoribosyltransferase (aprt; AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) to the aprt⁺ phenotype by means of DNA-mediated gene transfer. Transformation was effected by using unfractionated high molecular weight genomic DNA from Chinese hamster, human, and mouse cells and restriction endonuclease-digested DNA from rabbit liver. The transformation frequency observed was between 1 and 10 colonies per 10⁶ cells per 20 µg of donor DNA. Transformants displayed enzymatic activity that was donor derived as demonstrated by isoelectric focusing of cytoplasmic extracts. These transformants fall into two classes: those that are phenotypically stable when grown in the absence of selective pressure and those that are phenotypically unstable under the same conditions.

The DNA-mediated transfer of cellular genes, discovered (1) and previously exploited in the prokaryotes, has recently been extended to eukaryotes. Early studies on the transformation** of eukaryotic cells were restricted to viral genes (2, 3). Recently, transformation of yeast spheroplasts with recombinant DNA molecules has permitted the isolation and characterization of genes coding for selectable biochemical markers (4). In our laboratories we have transferred cellular genes from complex vertebrate genomes to cultured mammalian cells. In initial studies, we demonstrated transfer of the thymidine kinase (tk) gene of herpes simplex virus to mutant mouse Ltk⁻ cells (5). After optimizing the conditions for transformation in this model system, we were able to transfer the cellular tk gene by using unfractionated high molecular weight genomic DNA from various species as donors (6). The transformed cell expressed tk activity encoded by donor DNA.

The potential usefulness of this observation depends to a large extent on its generality. In principle, transformation should be detectable for all genes for which selection conditions are available. In this study, we demonstrate the transfer of the gene coding for adenine phosphoribosyltransferase (aprt; AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) to mutant cells lacking this enzyme (Ltk⁻ aprt⁻). Transformants express aprt activity with the characteristics of the organism from which the transforming DNA was derived. Taking these results together with our previous results, we have demonstrated that DNA-mediated gene transfer in animal cells can provide a bioassay for dominant-acting genes present at concentrations of one part per haploid genome.

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MATERIALS AND METHODS

Cell Culture. Murine Ltk⁻ aprt⁻ cells are derivatives of Ltk⁻ clone 1D cells (7) and were originally isolated and characterized by R. Hughes and P. Plagemann. They were generously provided by R. Hughes. Cells were maintained in growth medium [Dulbecco's modified Eagle's medium containing 10% calf serum (Flow Laboratories, Rockville, MD)] supplemented with diaminopurine at 50 µg/ml. Prior to transformation, cells were washed and grown for three generations in the absence of diaminopurine. HEP-2 (human), HeLa (human), CHO (Chinese hamster ovary), and Ltk⁻ cells were grown in growth medium. For CHO, medium was supplemented with 3X the usual concentration of nonessential amino acids. LH2b, a derivative of Ltk⁻ transformed with herpes simplex virus tk DNA, was maintained in growth medium containing hypoxanthine at 15 µg/ml, aminopterin at 0.2 µg/ml, and thymidine at 5.0 µg/ml (HAT) (5). All culture dishes were Nunclon (Vanguard International, Neptune, NJ) plastic.

Extraction and Restriction Endonuclease Cleavage of Genomic DNA. High molecular weight DNA was obtained from cultured cells (CHO, LH2b, and HeLa) or from frozen rabbit livers as previously described (6). High molecular weight salmon sperm DNA was obtained from Worthington. Restriction endonucleases were obtained from New England Biolabs. Restriction endonuclease cleavage (*Bam* I, *Hind* III, *Kpn* I, and *Xba* I) was performed in buffer containing 50 mM NaCl, 10 mM Tris-HCl, 5 mM MgCl₂, 7 mM mercaptoethanol, and bovine serum albumin at 100 µg/ml (pH 7.9). The enzyme-to-DNA ratio was at least two units/µg of DNA, and reaction mixtures were incubated at 37°C for at least 2 hr (one unit is the amount of enzyme that digests 1 µg of DNA in 1 hr). To monitor the completeness of digestion, 1 µl of nick-translated adenovirus-2 [³²P]DNA was incubated with 5 µl of reaction volume for at least 2 hr, cleavage products were separated by electrophoresis in 1% agarose gels, and digestion was monitored by exposing the dried gel to Cronex 2DC x-ray film.

Transformation and Selection. The transformation protocol was as described (8) with the following modifications. One day prior to transformation, cells were seeded at 0.7 × 10⁶ cells per

Abbreviations: tk, thymidine kinase; aprt, adenine phosphoribosyltransferase; HAT, hypoxanthine/aminopterin/thymidine.

[†] Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

[§] To whom correspondence should be addressed.

** We define transformation as a change in the genotype of a recipient cell mediated by the introduction of purified DNA. Transformation can frequently be detected by the stable and heritable change in the phenotype of the recipient cell that results from an alteration in either the biochemical or the morphological properties of the recipient.

dish. The medium was changed 4 hr prior to transformation. Sterile, ethanol-precipitated high molecular weight or restriction endonuclease-cleaved eukaryotic DNA dissolved in 1 mM Tris (pH 7.9)/0.1 mM EDTA was used to prepare DNA/CaCl₂, which contains DNA at 40 µg/ml and 250 mM CaCl₂ (Mallinkrodt). Twice-concentrated Hepes-buffered saline (2X HBS) was prepared; it contains 280 mM NaCl, 50 mM Hepes, and 1.5 mM sodium phosphate, pH adjusted to 7.10 ± 0.05. DNA/CaCl₂ solution was added dropwise to an equal volume of sterile 2X HBS. A 1-ml sterile plastic pipette with a cotton plug was inserted into the mixing tube containing 2X HBS, and bubbles were introduced by blowing while the DNA was being added. The calcium phosphate/DNA precipitate was allowed to form without agitation for 30–45 min at room temperature. The precipitate was then mixed by gentle pipetting with a plastic pipette, and 1 ml of precipitate was added per plate, directly to the 10 ml of growth medium that covered the recipient cells. After 4-hr incubation at 37°C, the medium was replaced and the cells were allowed to incubate for an additional 20 hr. At that time, selective pressure was applied. For tk⁺ selection, medium was changed to growth medium containing HAT. For aprt⁺ selection, cells were trypsinized and replated at lower density (about 0.5 × 10⁶ cells per 10-cm dish) in medium containing 0.05 mM azaserine and 0.1 mM adenine. For both tk⁺ and aprt⁺ selection, selective media were changed the next day, 2 days after that, and subsequently every 3 days for 2–3 weeks while transformant clones arose. Colonies were picked by using cloning cylinders and the remainder of the colonies were scored after formaldehyde fixation and staining with Giemsa. For characterization, clones were grown into mass culture under continued selective pressure. A record was kept of the apparent number of cell doublings for each clone isolated.

Enzyme Assays. Extracts were prepared by resuspending washed cell pellets (approximately 10⁷ cells) in 0.1 ml of 0.02 M potassium phosphate, pH 7, containing 0.5% Triton X-100. The supernatant (cytoplasm) obtained after 25 min of 700 × *g* centrifugation was used for the quantitation of enzymatic activity and for electrophoresis. aprt and protein were assayed as previously described (9). Inclusion of 3 mM thymidine triphosphate, an inhibitor of 5'-nucleotidase (10), in the reaction mixture did not increase AMP recovery, indicating that the nucleotidase was not interfering with the measurement of aprt activity. Isoelectric focusing of aprt was carried out essentially as described for hypoxanthine phosphoribosyltransferase (11) with the following exceptions: The polyacrylamide gel contained an Ampholine (LKB) mixture of 0.8% pH 2.5–4, 0.8% pH 4–6, and 0.4% pH 5–7. For assaying enzymatic activity, [2-³H]adenine [0.04 mM, 1 Ci/mmol, New England Nuclear (1 Ci = 3.7 × 10¹⁰ becquerels)] was substituted for hypoxanthine.

RESULTS

Transformation to the aprt⁺ Phenotype. Biochemical transformation occurs with low frequency and is usually detected by the ability of the rare transformed cell to grow under appropriate selective conditions. The development of a transformation system therefore requires a recipient cell that is both competent for transformation and sensitive to selection. In addition, the frequency at which the recipient cell spontaneously reverts to selection resistance must be lower than the frequency of transformation. Previous results indicated that Ltk⁻ cells (deficient for tk) are competent recipients for cellular DNA and undergo transformation to the tk⁺ phenotype at a rate of 1–10 colonies per 10⁶ cells per 20 µg of donor DNA (6). The aprt⁻ variant of Ltk⁻ cells (Ltk⁻ aprt⁻) grows in the presence of diaminopurine. aprt⁺ cells are selected in media containing

Table 1. Gene transfer with total genomic DNA from various species

Donor DNA	Total tk ⁺ colonies/ total plates	Average/ plate	Total aprt ⁺ colonies/ total plates	Average/ plate
Chinese hamster (CHO cells)	22/5	4.4	20/14	1.4
Human (HeLa cells)	42/4	10.5	95/14	6.8
Mouse (LH2b cells)	100/5	20.0	24/15	1.6
Salmon (testes)	0/5	0.0	0/15	0.0
None	0/5	0.0	0/15	0.0

High molecular weight DNA was prepared and coprecipitated with calcium phosphate; 20 µg of precipitated DNA (in 1 ml) was added to each plate. Transformants were scored for either the tk⁺ or aprt⁺ phenotype after selection.

azaserine and adenine. Azaserine blocks *de novo* purine biosynthesis, and adenine can be utilized for the synthesis of purine nucleotides only by aprt⁺ cells. Ltk⁻ aprt⁻ cells show a low rate of spontaneous reversion to the aprt⁺ phenotype as judged by their cloning efficiency in azaserine/adenine (unpublished studies).

Ltk⁻ aprt⁻ cells are therefore appropriate recipients for the transfer of the aprt gene. High molecular weight DNA was prepared from human, hamster, and wild-type mouse cultured cells. A calcium phosphate/DNA coprecipitate was added to Ltk⁻ aprt⁻ cells under a modification of the transformation conditions described by Graham and van der Eb (8). After 24 hr, cells were exposed to the selection media.

Colonies were scored after 2–3 weeks. The results of one experiment are shown in Table 1. The data demonstrate that transformation to the tk⁺ and aprt⁺ phenotypes can be effected with DNA preparations from hamster, human, and mouse. The frequency of transformation in each case was about 10 colonies per 10⁶ cells per 20 µg of DNA. No transformants resulted from treatment with salmon DNA and no aprt⁺ colonies arose in untreated cultures. This is in accord with the observation that the reversion of this line to aprt⁺ occurs with a frequency of 3 × 10⁻⁸ under these conditions (unpublished studies).

Individual transformant colonies were picked, in cloning cylinders, from separate plates to ensure that they represented independent transformation events. These colonies were grown

Table 2. aprt activities of parental and transformant clones

Cell line	Donor DNA	aprt activity, nmol AMP/min per mg
Donors		
HEp-2 (human)	—	2.19
CHO (Chinese hamster)	—	6.52
Ltk ⁻ (mouse)	—	2.09
Recipient		
Ltk ⁻ aprt ⁻	—	<0.005
Transformants		
MA-1	Mouse	1.94
MA-4	Mouse	2.29
HA-1	Human	1.72
HA-4	Human	1.37
CA-3	Chinese hamster	0.86
RA-1*	Rabbit	1.62

* This clone is a mouse cell revertant isolated after presumptive transformation with rabbit liver DNA.

into mass cultures under selective conditions (azaserine/adenine). Cytoplasmic extracts were prepared from individual clones and assayed for *aprt* activity. As shown in Table 2, *Ltk*⁻ *aprt*⁻ cytoplasmic extracts had negligible activity. In contrast, all transformants displayed enzymatic activities in the range of wild-type mouse *Ltk*⁻ cells.

Electrophoretic Characterization of *aprt*. Tables 1 and 2 indicate that the appearance of *aprt*⁺ colonies was dependent upon the addition of mammalian DNA, suggesting that gene transfer, rather than reversion, had occurred. Direct evidence for gene transfer was obtained by the electrophoretic characterization of the *aprt* in transformed clones. Isoelectric focusing in polyacrylamide gels clearly separates murine *aprt* from that of human, rabbit, and Chinese hamster (Fig. 1). Transformed clones derived from treatment with human or Chinese hamster DNA express *aprt*s with isoelectric points characteristic of the donor DNA species. No murine *aprt* activity is detected in these cells. In contrast, transformants derived after treatment with murine DNA express the murine *aprt* activity. These results argue strongly that the selected clones do not represent a special class of revertants that reexpress the parental murine *aprt* rather than the donor DNA *aprt*. Reversion of the parental *Ltk*⁻ *aprt*⁻ clone can occur, however, as indicated in one experiment in which rabbit DNA was used as donor. In this case, the *aprt*⁺ clone subsequently isolated exhibited murine *aprt* activity.

Stability of the Transformed Phenotype. We next asked if expression of *aprt* was stable in the absence of selective pressure. Individual transformant clones, grown into mass culture under

Table 4. Gene transfer with restriction endonuclease-cleaved DNA

Endonuclease	Total <i>aprt</i> ⁺ colonies/ total plates	Average/ plate
<i>Bam</i> I	1/9	0.1
<i>Hind</i> III	104/10	10.4
<i>Kpn</i> I	109/8	13.6

High molecular weight rabbit liver DNA was prepared and cleaved to completion with the indicated restriction endonucleases. Cleaved DNA was used as donor in transformation experiments.

selective pressure, were subcultured for various times in the absence of selective pressure. The fraction of cells that retained the *aprt*⁺ phenotype was determined by measuring cloning efficiencies in selective and nonselective media. The results of these experiments (Table 3) demonstrate that transformants fall into two categories: stable transformants that retain the ability to grow in azaserine/adenine when cultured in the absence of selective pressure (HA-1, HA-4, CA-3); and unstable transformants that do not (CA-1, MA-1, MA-4). The rate of loss of the *aprt* phenotype can be calculated from these data if one assumes that rate of loss is constant in each generation (see footnote §, Table 3). For the parental *Ltk*⁻ (*aprt*⁺) cells, a revertant of *Ltk*⁻ *aprt*⁻ (RA-1), and the stable transformants, the rate of loss was no more than 2% per generation. For the unstable transformants, the calculated rate of loss was as high as 27% per generation (CA-3).

Table 3. Stability of the transformed phenotype

Cell line*	Exp.	Generations in†		Relative cloning efficiency in selective medium‡	Rate of loss of <i>aprt</i> ⁺ phenotype per generation§
		Selective medium	Neutral medium		
<i>Ltk</i> ⁻	1	23	3	0.89	
	2	0	26	0.93	<0.01
CA-1	1	37	2	0.52	
	2	24	16	0.006	0.27
CA-3	1	42	3	0.96	
	2	25	20	0.72	0.02
HA-1	1	64	3	1.00	
	2	47	20	0.84	<0.01
HA-4	1	41	3	0.64	
	2	25	20	0.76	<0.01
MA-1	1	39	4	0.28	
	2	24	26	0.01	0.14
MA-4	1	38	4	0.23	
	2	24	23	0.01	0.15
RA-1	1	47	3	0.88	
	2	31	20	0.96	<0.01

* *Ltk*⁻ is the parental line for *Ltk*⁻ *aprt*⁻. RA-1 is a revertant *aprt*⁺ derivative of the latter. All other lines represent *aprt*⁺ transformants of *Ltk*⁻ *aprt*⁻. For derivatives and enzyme characterization see Table 1 and Fig. 1.

† Clones were picked and grown in selective medium for a known number of generations. Cells were then grown in neutral medium for a known number of generations prior to measuring their cloning efficiencies under selective and nonselective conditions.

‡ One hundred cells were plated in triplicate into selective (azaserine/adenine) and nonselective media. The relative cloning efficiency in selective medium is defined as the ratio of the cloning efficiency under selective conditions to the cloning efficiency under nonselective conditions. The latter was generally 50–70%.

§ In these calculations we have assumed that for any given cell line the rate of loss of the *aprt* phenotype is constant in each generation. With that assumption, the rate of loss per generation may be calculated by using the formula $F_M(1 - X)^{N-M} = F_N$, in which F_M is the relative cloning efficiency in selective medium after M generations in nonselective medium; F_N is similarly defined; and X is the rate of loss per generation.

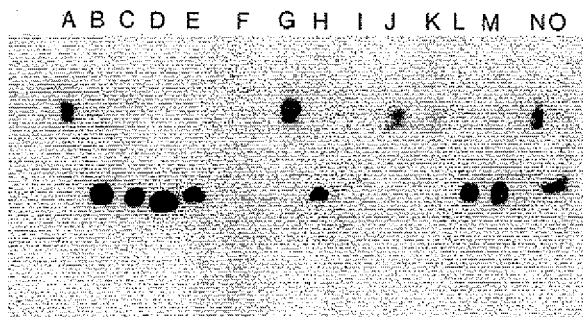


FIG. 1. Isoelectric focusing of aprt. The high-speed supernatants from homogenates of wild-type and mutant cells, transformed cells, and rabbit liver were focused on 4.5% acrylamide gels containing an Ampholine mixture of 0.8% pH 2.5–4/0.8% pH 4–6/0.4% pH 5–7. For development of enzyme activity, $[2\text{-}^3\text{H}]$ adenine was used and the product was blotted onto polyethyleneimine-cellulose and localized by fluorography. A, Ltk⁻ cell extract; B, rabbit liver homogenate; C, HEp-2 cell extract; D, CHO cell extract; E, extract of Ltk⁻ aprt⁻ cells transformed with HeLa cell DNA (HA-1); F, extract from cells transformed with CHO cell DNA (CA-3); G, extract from cells transformed with LH2b cell DNA (MA-1); H, extract from cells transformed with HeLa cell DNA (HA-4); I, extract from cells transformed with CHO cell DNA (CA-1); J, extract from cells transformed with LH2b cell DNA (MA-4); K, extract from an Ltk⁻ aprt⁻ revertant (RA-1); L, extract from HEp-2 cells; M, extract from CHO cells; N, extract from Ltk⁻ cells; O, extract from rabbit liver homogenate.

Transformation with Restriction Endonuclease-Cleaved DNA. It was of interest to determine whether transformation of aprt⁻ cells could be performed with restriction enzyme fragments of DNA. This requires the use of restriction endonucleases that do not cleave the aprt gene. High molecular weight DNA was therefore prepared from rabbit liver and digested to completion with a variety of restriction enzymes. This restriction endonuclease-cleaved DNA was used in transformation assays. The data are summarized in Table 4. Although *Bam* I destroyed the ability of DNA to transfer aprt, cleavage of rabbit DNA with either *Kpn* I or *Hind*III did not result in a reduction in transformation efficiency compared with the efficiency obtained when the transformation was performed with uncleaved DNA. Subsequent experiments indicated that cleavage of rabbit DNA with *Xba* I also does not destroy the aprt gene (data not presented).

DISCUSSION

In this study we have demonstrated the transformation of aprt⁻ mouse cells to aprt⁺, using both high molecular weight and restriction endonuclease-cleaved DNA from a variety of species as donors. Stable transformation appears at a frequency 100-fold higher than the spontaneous reversion frequency displayed by this cell line. The frequency of transformation ranges from 1 to 10 colonies per 10^6 cells per 20 μg of donor DNA. These results together with comparisons of the aprt activities from donors and transformants by isoelectric focusing directly demonstrate that the aprt in transformants is derived from the donor

DNA and is not due to reversion or reactivation of the murine gene.

The transformants we have isolated fall into two categories: those that retain the ability to express aprt even in the absence of selective pressure and those that do not. In this respect, biochemical transformants obtained after DNA-mediated gene transfer resemble transformants obtained after chromosome-mediated transfer (12, 13). The molecular basis for the observed phenotypic instability is not known at the present time.

The method employed to transfer both the tk and aprt genes can in principle be applied to any gene for which appropriate selective conditions and recipient cells exist. We have, for example, recently succeeded in transferring the gene coding for a methotrexate-resistant folate reductase gene (14) to wild-type cells (unpublished results). The generality of these observations indicates that transformation will facilitate the dissection of complex cellular phenotypes in eukaryotic cells.

To date, the isolation of genes from animal cells has been confined to those loci for which hybridization probes are available. Numerous interesting loci, however, are not transcribed in amounts sufficient to generate hybridization probes. DNA-mediated gene transfer provides a unique bioassay for gene function and a method that could be employed in alternative approaches for gene isolation.

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Biochemistry

Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase

(pBR322-simian virus 40 DNA vectors/transformation of animal cells/integration of DNA/xanthine phosphoribosyltransferase)

R. C. MULLIGAN* AND P. BERG

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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ABSTRACT Cultured monkey (TC7) and mouse (3T6) cells synthesize an *Escherichia coli* enzyme, xanthine-guanine phosphoribosyltransferase (XGPRT; 5-phospho- α -D-ribose-1-diphosphate:xanthine phosphoribosyltransferase, EC 2.4.2.22), after transfection with DNA vectors carrying the corresponding bacterial gene, *Ecogpt*. In contrast to mammalian cells, which do not efficiently use xanthine for purine nucleotide synthesis, cells that produce *E. coli* XGPRT can synthesize GMP from xanthine via XMP. After transfection with vector-*Ecogpt* DNAs, surviving cells producing XGPRT can be selectively grown with xanthine as the sole precursor for guanine nucleotide formation in a medium containing inhibitors (aminopterin and mycophenolic acid) that block *de novo* purine nucleotide synthesis. Cells transformed for *Ecogpt* arise with a frequency of 10^{-4} to 10^{-5} ; they appear to be genetically stable in as much as there is no discernible decrease in XGPRT formation or loss in their ability to grow in selective medium after propagation in nonselective medium. Although several of the vector-*gpt* DNAs can replicate in monkey and mouse cells, none of the transformants contain autonomously replicating vector-*gpt* DNA. Rather, the *gpt* transformants contain one to five copies of the transfecting DNA associated with, and most probably integrated into, cellular DNA sequences. In several transformants, vector-coded gene products for which there was no selection are also synthesized. This suggests that recombinant DNAs containing *Ecogpt* as a selective marker may be useful for cotransformation of nonselectable genes.

Considerable progress has been made in developing procedures for the introduction, propagation, and maintenance of selected genes in mammalian cells (1-5). One objective of these studies is to obtain an assay for the function of isolated genes *in vivo*. Such an assay, along with current techniques for gene cloning, *in vitro* mutagenesis, and rapid DNA sequence determination, could uncover additional correlations between the expression and regulation of genes and their structure and chromosomal organization. The availability of general transducing vectors would also permit the construction of novel cellular genomes, a capability that has proved valuable in physiologic and genetic studies of prokaryote organisms.

We have used simian virus 40 (SV40) as a transducing vector; specifically, regions of the SV40 genome have been replaced *in vitro* by selected DNA segments, and the resulting recombinant DNAs have been propagated as viruses with complementing helpers in cultured monkey cells (2, 6). But this experimental design has several shortcomings: (i) Only DNA segments smaller than 5 kilobases (kb) can be transduced in this way; (ii) the host cell is killed during the course of the infection, precluding the opportunity to monitor the transduced gene's expression in continuously multiplying cells; (iii) studies are limited to hosts permissive to SV40, a constraint that excludes many specialized and differentiated animal cells as recipients of the transduced genes.

To circumvent these disadvantages, we sought to develop transducing vectors that can be introduced into a wide variety of mammalian cells and maintained indefinitely, either as autonomously replicating or as stably integrated genetic elements. Because transfection of mammalian cells with DNA is inefficient (1, 7), the recovery of transformants without a selection is impractical. Therefore, our first goal was to obtain a gene whose expression in the transduced cells would allow them to be grown selectively. That purpose has been achieved by the isolation of a gene from *Escherichia coli* (*Ecogpt*) that encodes xanthine-guanine phosphoribosyltransferase (XGPRT; 5-phospho- α -D-ribose-1-diphosphate:xanthine phosphoribosyltransferase, EC 2.4.2.22), a purine salvage-pathway enzyme.

E. coli XGPRT and the analogous mammalian enzyme, hypoxanthine phosphoribosyltransferase (HPRT; IMP pyrophosphate phosphoribosyltransferase; EC 2.4.2.8), catalyze the conversion of hypoxanthine and guanine to IMP and GMP, respectively; the bacterial enzyme also efficiently converts xanthine to XMP (8), a reaction catalyzed only very poorly by the mammalian enzyme (9). We have previously reported that infection of cultured mammalian cells with recombinant DNAs containing the *Ecogpt* segment induces the synthesis of bacterial XGPRT (5). Moreover, HPRT-negative cell lines transfected with appropriate vectors containing the *Ecogpt* gene synthesize XGPRT and grow selectively in hypoxanthine/aminopterin/thymidine medium (5). This finding suggests that *E. coli* XGPRT can provide the purine salvage function of mammalian HPRT. On that premise, we have devised a procedure that permits the selective recovery of *Ecogpt* transformants from among normal nontransformed cells. The selection relies on the fact that *Ecogpt* transformed cells, but not normal cells, can use xanthine to overcome an inhibition of the *de novo* synthesis of GMP.

METHODS

Cell Culture and Selection for Transformants. African green monkey kidney cells (TC7) and mouse fibroblasts (3T6) were maintained in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, and 5% fetal calf serum. Twenty-four hours after seeding approximately 10^6 cells on 100-mm plates, the cultures were transfected with 10-20 μ g of one of the plasmid-*Ecogpt* DNAs according to Parker and Stark's modification (7) of Graham and Van der Eb's procedure (1), except that carrier salmon sperm DNA was omitted.

After 3 days at 37°C in Eagle's medium containing 5% fetal calf serum, the transfected cell monolayers were treated with

Abbreviations: SV40, simian virus 40; kb, kilobase(s); XGPRT, xanthine-guanine phosphoribosyltransferase; GPT, guanine phosphoribosyltransferase; HPRT, hypoxanthine phosphoribosyltransferase; T and t, SV40 large and small tumor antigens, respectively; APRT, adenine phosphoribosyltransferase; DHFR, dihydrofolate reductase; TK, thymidine kinase.

* Present address: Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139; and Endocrine Unit, Massachusetts General Hospital, Boston, MA 02114.

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trypsin-EDTA, and $\approx 5 \times 10^5$ cells were dispersed on 100-mm plates in Eagle's medium containing 10% dialyzed fetal calf serum/xanthine (250 $\mu\text{g}/\text{ml}$)/hypoxanthine (15 $\mu\text{g}/\text{ml}$) or adenine (25 $\mu\text{g}/\text{ml}$)/L-glutamine (150 $\mu\text{g}/\text{ml}$)/thymidine (10 $\mu\text{g}/\text{ml}$)/aminopterin (2 $\mu\text{g}/\text{ml}$)/mycophenolic acid (25 $\mu\text{g}/\text{ml}$). Mycophenolic acid was prepared in 0.1 M NaOH and neutralized with 0.1 M HCl [Ham's F12 medium (GIBCO) supplemented with the same concentrations of xanthine, aminopterin, and mycophenolic acid has also served successfully for the selection]. Twenty-four hours later, the culture fluid was replaced with fresh medium containing the same supplements; thereafter, it was changed every 3 days. Colonies were readily visible in 7–10 days and isolated with cloning cylinders after 14 days. The number of *Ecogpt* transformants was determined by counting colonies after fixation in methanol and staining with 10% Giemsa; transformation frequency is expressed as the fraction of cells plated that grow under the selective conditions.

Mycophenolic acid alone is sufficient to prevent extensive growth of the cells that have been tested, but in some cell lines the block appears to be delayed and initially incomplete. The addition of aminopterin blocks *de novo* synthesis of all purines and, even in the presence of hypoxanthine or adenine to provide for AMP formation (see Fig. 2), the dependence on xanthine for GMP formation is more exacting. Optimal concentrations of inhibitors and supplements for efficient inhibition and restoration of growth, respectively, should be determined for each cell line used. The optimal lethal concentration of mycophenolic acid differs for different cell lines and can be determined by titration in the presence and absence of guanine.

DNA Extraction and Hybridization Procedures. Plasmid DNAs were isolated from *E. coli* as described (10) and centrifuged twice to equilibrium in cesium chloride/ethidium bromide gradients (11). High and low molecular weight DNAs were obtained from cloned cultures of the transformants as described by Wigler *et al.* (12) and Hirt (13), respectively; the Hirt supernatants were extracted once with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1) and then treated with ethanol to precipitate the DNA. Approximately 10 μg of high molecular weight transformed-cell DNA was cleaved with restriction endonuclease, subjected to electrophoresis on agarose gels, transferred to diazobenzoyloxymethyl or nitrocellulose paper (14–16), and annealed with ^{32}P nick-translated DNA (17) containing the *Ecogpt* sequence ($0.7\text{--}2 \times 10^6$ cpm/ μg).

Enzyme and T-Antigen Assays. The preparation and assay of cell extracts for purine phosphoribosyltransferase has been described (5). To measure the formation of SV40 tumor antigens in various transformants, cells were labeled for 1 hr at 37°C with 200 μCi of [^{35}S]methionine (1000 Ci/mmol) in 1 ml of medium containing 4% dialyzed fetal calf serum but lacking methionine. Extracts (6) were treated with hamster anti-SV40 T-antigen serum (Flow Laboratories, Rockville, MD), the immune complexes were subjected to electrophoresis on 10–15% gradient NaDodSO₄/acrylamide gels, and the labeled protein bands were visualized by autoradiography.

RESULTS

Transfecting Vectors for *Ecogpt*. A set of plasmid DNA vectors that promote the expression of suitably positioned genes or cDNAs after their introduction into cultured animal cells has been developed (Fig. 1). pSV2, the prototype, contains a cDNA or other coding sequence of interest, downstream (3'-proximal) from the SV40 early transcription promoter (at SV40 ori) and upstream (5'-proximal) from a DNA segment that ensures splicing and polyadenylation of the ensuing transcript (18). pSV3 and pSV5 are derivatives of pSV2 that contain an intact SV40 or polyoma early region, respectively, inserted at a *Bam*HI restriction site. Each of the vectors contains a DNA segment from

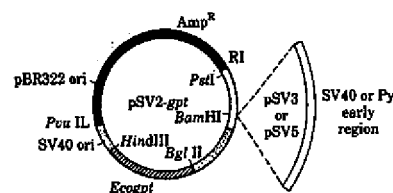


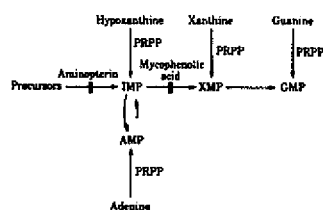
FIG. 1. Structure of pSV-gpt vectors. All vectors are shown with the *gpt* segment hatched. The solid black segment represents the 2.3 kb of pBR322 DNA sequences containing the origin of pBR322 DNA replication and the ampicillinase gene. SV40 sequences in pSV2 are shown as lightly stippled regions and include a segment containing the SV40 origin of DNA replication (ori) and the early region promoter (0.71–0.65 map units), the small tumor-antigen intervening sequence (0.56–0.44 map units), and the sequence at which termination and polyadenylation of SV40 early transcripts (0.19–0.1 map units) occurs. pSV3 and pSV5 DNAs contain, in addition, sequences encoding the early region of SV40 and polyoma, respectively.

the bacterial plasmid pBR322, which enables them to be amplified in *E. coli*. This design permits the transduction of larger DNA segments than can be accommodated with virus genomes as vectors. In addition, the manipulations and expense associated with the construction and propagation of particular recombinant DNAs are greatly reduced. The vectors pSV2, pSV3, and pSV5 containing the cloned segment of *Ecogpt* DNA (ref. 5; see Fig. 1) were used to develop the selection protocol for cells containing *E. coli* XGPRT.

Rationale for Selection of Cells Expressing *E. coli* XGPRT. Purine nucleotides are synthesized *de novo* or by salvage pathways (ref. 19; Fig. 2). In the *de novo* pathway, IMP, the first nucleotide intermediate, is converted to AMP via adenylosuccinate and to GMP via XMP. Salvage of free purines occurs by condensation with phosphoribosyl pyrophosphate: Adenine phosphoribosyltransferase (APRT) accounts for the formation of AMP from adenine, HPRT converts hypoxanthine and guanine to IMP and GMP, respectively. There appears to be no mammalian enzyme comparable to the bacterial enzyme for converting xanthine to XMP (9).

Mycophenolic acid, an inhibitor of IMP dehydrogenase (20), prevents the formation of XMP and, therefore, of GMP. The inhibition of purine nucleotide synthesis can be made even more pronounced by the addition of aminopterin, which prevents the *de novo* synthesis of IMP (21). Supplementing the medium with either hypoxanthine or adenine does not reverse the effect of the two inhibitors. However, the inhibition of cell growth by these compounds can be reversed by adding guanine and either hypoxanthine or adenine to the medium, because these bases can be converted to their respective mononucleotides by purine phosphoribosyltransferases. Indeed, the plating efficiency of monkey and mouse cells in medium containing both inhibitors is nearly the same as in normal medium, if adenine and guanine (each at 25 $\mu\text{g}/\text{ml}$) are added. Because normal mammalian cells convert xanthine to XMP very poorly, they cannot grow if medium containing aminopterin and mycophenolic acid is supplemented with adenine and xanthine. However, cells that contain *E. coli* XGPRT grow under these conditions.

FIG. 2. Pathways of purine nucleotide synthesis and sites of inhibition by aminopterin and mycophenolic acid. Aminopterin blocks *de novo* synthesis of purines from precursors at two steps involving formylation (19). Mycophenolic acid specifically inhibits the conversion of IMP to XMP by IMP dehydrogenase (20). PRPP, phosphoribosyl pyrophosphate.



Transformation of Monkey and Mouse Cells with pSV2-, pSV3-, or pSV5-*gpt* DNAs. About 10^6 monkey (TC7) or mouse (3T6) cells were transfected with 10–20 μ g of pSV2, pSV3-, or pSV5-*gpt* DNA; after 3 days in nonselective medium, the cells were treated with trypsin and replated in medium containing aminopterin and mycophenolic acid and supplemented with xanthine and either hypoxanthine or adenine. Cells that received no DNA or pSV2 containing a rabbit β -globin cDNA in place of the *Ecogpt* segment did not survive under the selective conditions; cells transfected with pSV2-, pSV3-, or pSV5-*gpt* DNA yielded 2–25 surviving clones per 10^5 cells plated, transformation being about 5 times more efficient with 3T6.

Several representative cloned TC7 or 3T6 colonies transformed with each of the vectors were subcultured in the selective medium for further study. Selected transformants were assayed for xanthine phosphoribosyltransferase activity by their ability to convert [14 C]xanthine to XMP (5). Transformed cell extracts consistently gave 2–8 times higher values than those of comparable extracts from their untransformed progenitors (Table 1). Also, bacterial and mammalian guanine phosphoribosyltransferase (GPRT) activities can be distinguished by the latter's sensitivity to inhibition by hypoxanthine; 20 μ M of guanine plus 400 μ M of hypoxanthine virtually completely inhibits mammalian GPRT activity, but has little or no effect on that of *E. coli* (5). Thus, GMP formation in the absence of hypoxanthine measures the sum of the cellular and the bacterial GPRT activities whereas measurement in its presence assays the bacterial activity alone. Thus, in extracts of *Ecogpt*-transformed cells, 15–40% of the GPRT activity is not inhibited by hypoxanthine, a property characteristic of the *E. coli* GPRT (Table 2). The electrophoretic mobility of *E. coli* XGPRT differs from that of the mammalian HPRT (5). Clearly, extracts of *Ecogpt*-transformed TC7 and 3T6 cells possess GPRT activities with electrophoretic mobilities characteristic of both the bacterial and the mammalian enzymes (Fig. 3). We conclude that the newly acquired ability of the transformants to convert xanthine to XMP and, thereby, grow in the selective conditions used results from the formation of XPRTP by expression of the *Ecogpt* gene.

Stability of *Ecogpt*-Transformed Clones in the Absence of Selection. Several independent *Ecogpt* transformants have been propagated in the absence of mycophenolic acid and aminopterin for about 75 generations. For each line examined so far, after 0, 30, and 75 generations in nonselective media, the ratio of the cells' plating efficiency in selective vs. nonselective media is 1.0 ± 0.2 . This suggests that few, if any, of the transformants lose the ability to grow in the selective medium during subculture in the absence of selection for *Ecogpt* expression. Moreover, when extracts of cells grown for 75 generations without selection were assayed for bacterial GPRT activity, the amount was not substantially different from that of cells maintained continuously in the selective medium (data not shown).

Expression of SV40 T Antigens in pSV3-*gpt* Transformants. Of particular interest is whether an unselected genetic marker

Table 2. Detection of *E. coli* GPRT activity in extracts of transformants in absence and presence of hypoxanthine

Cell extract	GPRT activity		A/B
	Absence (B)	Presence (A)	
TC7	2.0	—	—
TC7/pSV2- <i>gpt</i>	1.6	0.31	0.19
TC7/pSV3- <i>gpt</i>	2.5	0.42	0.17
TC7/pSV5- <i>gpt</i>	2.5	0.34	0.14
3T6	1.0	0.08	—
3T6/pSV2- <i>gpt</i>	0.66	0.29	0.36
3T6/pSV3- <i>gpt</i>	0.95	0.24	0.17
3T6/pSV5- <i>gpt</i>	1.3	0.60	0.38

To calculate A/B, background GPRT activity in nontransformed 3T6 extracts in the presence of hypoxanthine (8% of B) was subtracted from the +GPRT activity of the 3T6 transformants.

associated with the vector-*gpt* DNA can be expressed in the transformants. This was examined by testing for the presence of SV40 large and small tumor (T and t, respectively) antigens in representative TC7 and 3T6 cells transformed with pSV3-*gpt* as described in *Methods* (Fig. 4). None of the pSV3-*gpt*-transformed TC7 cells examined so far (four independent isolates) produce T antigen. However, both pSV3-*gpt*-transformed 3T6 cell extracts examined contain proteins that migrate more slowly than SV40 T antigen; whether these proteins are related to T antigen remains to be determined. A band migrating with t antigen is produced in two of the four pSV3-*gpt*-transformed TC7 clones and in both pSV3-*gpt* transformed 3T6 clones (Fig. 4). Thus, in several instances, two linked genetic functions—the *Ecogpt* and an SV40 early gene—appear to be cotransduced.

Physical State and Number of Vector-*gpt* DNA Copies in Transformants. An important question about the transformants is whether *Ecogpt*, and the associated vector DNA sequences, are integrated into the host cells' chromosomal DNA or reside as free, autonomously replicating, plasmid-like elements. We tested the latter possibility by preparing extracts of representative TC7 and 3T6 transformed cells according to Hirt's method (13) and subjecting them to electrophoresis on agarose gels. DNA imprints on diazobenzyloxymethyl (14, 15) or nitrocellulose (16) were hybridized with 32 P-labeled *Ecogpt* DNA and autoradiographed. Comparable Hirt extracts from nontransformed TC7 or 3T6 extracts, supplemented with an equivalent of 0.5, 5, or 50 pSV3-*gpt* DNA copies per cell, were analyzed on the same gels for comparison (Fig. 5). There were no de-

Table 1. Detection of *E. coli* xanthine phosphoribosyltransferase activity in extracts of *Ecogpt*-transformants

Cell extract	XMP formed*
TC7	1.1
pSV2/TC7	2.8
pSV3/TC7	4.8
pSV5/TC7	3.6
3T6	0.6
pSV2/3T6	2.0
pSV3/3T6	1.5
pSV5/3T6	4.9

* Expressed as (nmol/min)/mg of protein.

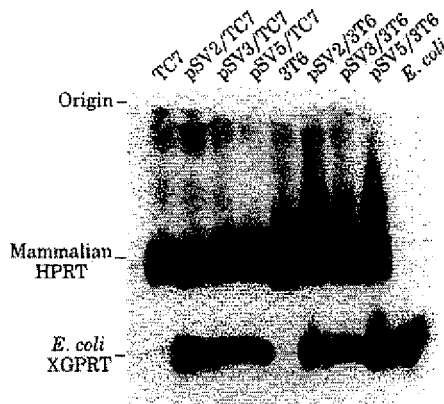


FIG. 3. Detection of GPRT in protein extracts prepared from pSV-*gpt* transformants by *in situ* assay after gel electrophoresis. The electrophoresis of cell extracts and *in situ* assays of GPRT activity have been described (5).

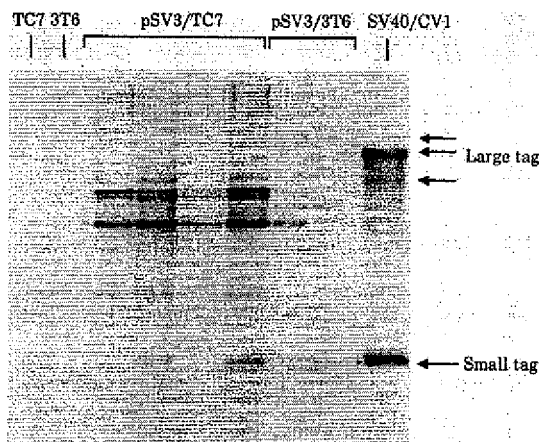


FIG. 4. Immunoprecipitation of SV40 tumor antigens produced in pSV3-*gpt* transformants. Extracts corresponding to 3×10^5 cells were prepared from the transformants and analyzed as described in *Methods*. The first two tracks represent extracts from untransformed TC7 and 3T6 cells and the last from SV40 infected CV1 cells. The other extracts are from independent pSV3-*gpt* transformed TC7 or 3T6 cells.

tectable vector-*gpt* DNA sequences in the Hirt extracts from either pSV3-*gpt*-transformed TC7 cells or pSV5-*gpt*-transformed 3T6 cells. This suggests that vector-*Ecogpt* DNA is most probably not maintained in the transformants as autonomously replicating species resembling the transfecting plasmids.

By contrast, *Ecogpt*-transformed cells contain vector-*Ecogpt* DNA sequences associated with high molecular weight chromosomal DNA. Fig. 6 left shows the results of hybridizations of restriction endonuclease-cleaved cell DNAs from various TC7 and 3T6 transformants with a 32 P-labeled *Ecogpt* DNA probe. *Sac* I does not cleave pSV2-*gpt* DNA; consequently, with cellular DNA from pSV2-*gpt* transformants, each hybridizing band represents at least one copy of all or part of a transfecting DNA sequence embedded in the cellular DNA. *Eco*RI cleaves both pSV2- and pSV3-*gpt* DNAs only once; therefore, a single, integrated, uninterrupted vector-*gpt* DNA segment should yield two bands, each containing a segment of vector-*gpt* DNA

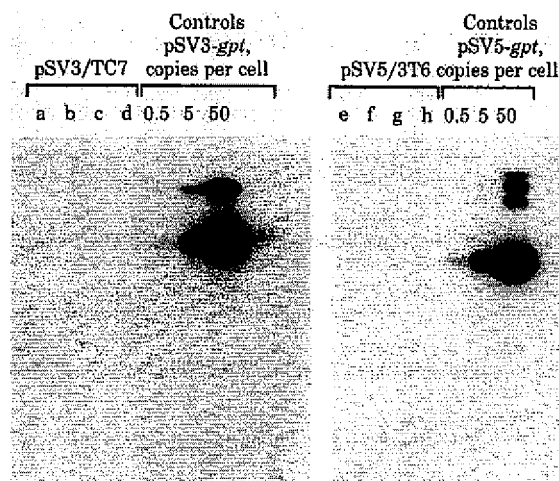


FIG. 5. Analysis of "free" vector DNA in pSV3-*gpt*-transformed TC7 and pSV5-*gpt*-transformed 3T6 cells. Each lane contains Hirt supernatant from 5×10^5 cells. Lanes: a-d, supernatant from pSV3-*gpt*-transformed TC7 cells; e-h, supernatant from pSV5-*gpt*-transformed 3T6 cells. Controls, supernatants from normal cells supplemented with pSV3-*gpt* or pSV5-*gpt* DNAs at 0.5, 5, or 50 equivalents.

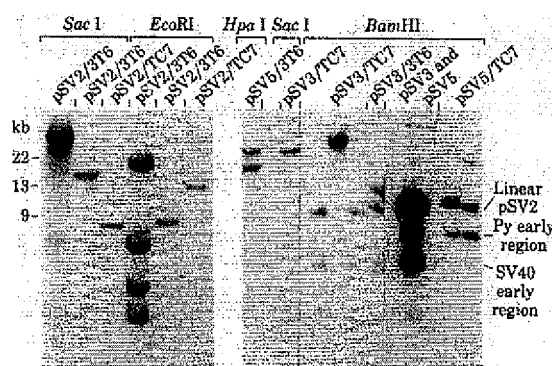


FIG. 6. Vector-*Ecogpt* DNA sequences in the high molecular weight DNA extracted from pSV-*gpt* transformants. (Left) Cell DNA from transformants was digested with *Sac* I or *Eco*RI endonucleases, subjected to electrophoresis in 1% agarose and transferred to diazobenzyloxymethylcellulose paper. The imprints were hybridized with nick-translated 32 P-labeled pSV2-*gpt* DNA. Size markers were from *Bgl* II endonuclease cleaved phage λ DNA. (Right) Cell DNA from pSV3-*gpt* and pSV5-*gpt* transformants was digested with *Sac* I, *Hpa* I, or *Bam*HI and analyzed in left, except that transfer was to nitrocellulose paper. The hybridization probe was a mixture of 32 P-labeled pSV3-*gpt* and pSV5-*gpt* DNAs. Size markers were from a mixture of *Bam*HI-cleaved pSV3-*gpt* and pSV5-*gpt* DNAs (≈ 100 pg of each).

plus flanking cellular DNA sequences. A tandemly repeated or very closely spaced arrangement would yield a band containing full-length vector-*gpt* DNA.

Sac I or *Eco*RI digestion of the DNAs extracted from several pSV2-*gpt* transformants of 3T6 and TC7 cells suggests that the number of vector-*gpt* copies per transformed cell genome is small (in the 1-5 range). Integration of a vector-*gpt* copy near its *Eco*RI restriction site or deletion of that restriction site could account for the occurrence of only one labeled band after digestion of two of the cell DNAs with *Eco*RI.

Conceivably, pSV3-*gpt*-transformed TC7 and pSV5-*gpt*-transformed 3T6 cells might contain a larger number of vector-*gpt* DNA copies per genome because the transfecting DNAs can replicate in these hosts (unpublished). However, the *Sac* I digest of a pSV3 transformant and the *Hpa* I digest of a pSV5 transformant indicate this is not the case (Fig. 6 right).

Another question concerns the co-integration of nonselected segments of the transfecting DNA (i.e., those sequences unrelated to *Ecogpt* expression): Are they retained, and, if so, are they intact? We examined this point by digesting cell DNAs from several pSV3-*gpt* and pSV5-*gpt* transformants with *Bam*HI and analyzing the products as described above. Because pSV3-*gpt* and pSV5-*gpt* contain the SV40 and polyoma early regions, respectively, at the *Bam*HI restriction site of pSV2 (Fig. 1), cleavage of pSV3- and pSV5-transformed cell DNAs with *Bam*HI should give a full-length pSV2-*gpt* segment or a full-length virus early-region segment, depending on where the recombination between the vector-*gpt* and the host DNA occurred. This analysis shows that such nonselected segments can indeed be co-integrated in an intact form (Fig. 5 right), but additional transformants need to be examined, particularly by molecular cloning of the integrated vector-*gpt* DNA segments, to find the precise organization of the integrated copies and their correspondence to the arrangement in the transfecting DNAs.

DISCUSSION

Stable genetic transformation of mammalian cells after transfection with DNA is a relatively rare event; consequently, the recognition and recovery of rare transformants requires a selection for the appropriate phenotype. For example, oncogenic

transformants arising from transfection with viral DNAs can be selected by their enhanced tumorigenicity or by changes in their growth characteristics in culture (22, 23). Transformation induced by DNAs coding for thymidine kinase (TK) (4, 12, 24) and dihydrofolate reductase (DHFR) (unpublished results), as well as for APRT (25) and HPRT (26, 27) has been detected by complementation of the respective defects in variant cell lines. Additionally, growth in the presence of methotrexate *in vitro* (28) has been used to monitor transformation of normal cells with DNA containing DHFR genes. More recently, transformation for human β -globin (29) and chicken ovalbumin (30) genes, neither one of whose functions can be selected for directly, has been achieved by cotransfection with DNA containing TK or DHFR genes as the selectable marker.

A principal shortcoming of present selection systems is the necessity for specific mutant cell lines as recipients of the transforming DNA. Thus, transformation or cotransformation relying on genes coding for TK, APRT, or HPRT as selective markers requires TK⁻, APRT⁻, or HPRT⁻ cell lines, respectively, for their detection. The scarcity of such mutants among specialized cell types makes a variety of potentially interesting experiments difficult—e.g., the introduction of globin genes into precursor and mature erythroid cells or of chicken ovalbumin genes into steroid sensitive oviduct cells. Dominant-acting genetic markers—e.g., those that can produce a discernible change in the phenotype of normal cells—offer a way out of this difficulty. The isolation of methotrexate-resistant transformants after transfection of normal cells with DNA from drug-resistant cells (28) exemplifies this approach; however, in this instance, the transformation frequency is low and its utility for cotransformation of other genes is limited. Our experiments suggest that *Ecogpt* provides an alternative dominant selective marker; the expression of this gene confers a novel capability on mammalian cells—efficient utilization of xanthine for GMP formation—and, thereby, a means for its selection in normal cells. Thus far, all of the cell lines tested [monkey (TC7, CV1), mouse (3T6, MEL), human (Lesch-Nyhan), and hamster (CHO)] fail to grow in the selective medium, suggesting that transformation and cotransformation using *Ecogpt* as the selective marker is probably feasible in a wide variety of cells.

Although pSV3- and pSV5-*gpt* DNAs replicate in TC7 and 3T6 cells, respectively (unpublished results), the transformation frequency with the permissive pairings was essentially the same as with the nonreplicating vector pSV2-*gpt* DNA or with a non-permissive pairing (i.e., pSV3/3T6). Moreover, vector-*gpt* DNAs that do replicate in the transfected hosts do not yield transformants containing free or autonomously replicating vector-*gpt* DNAs. Both observations could be accounted for if the transforming event *per se* is not influenced by the replicability or increased copy number of the vector-*gpt* DNA. However, a more likely possibility is that transformants that have autonomously replicating vector-*gpt* DNA are selected against, perhaps because such autonomous replication competes with the cellular genome for limiting replication functions or because elevated levels of XGPRT and its metabolites disrupt cellular metabolism. A necessity to prevent replication and ensure stable integration of pSV3-*gpt* and pSV5-*gpt* DNAs in their respective permissive hosts would account for the absence of T antigens in these transformants. Formation of SV40 T antigen in several pSV3-*gpt*-transformed 3T6 clones can be explained by the fact that the transfecting pSV3-*gpt* DNA does not replicate in 3T6 mouse cells.

The existence of several restriction sites in pSV2-*gpt* DNA and its derivatives [e.g., *Pst*I, *Eco*RI, and *Bam*HI (see Fig. 1)] provides a way to cotransform a variety of cells with other genes of interest. For example, DNA segments containing the human globin (31), chick ovalbumin (32), or hormone gene (33) families

can be inserted into the vectors and then into appropriate cells by using the *Ecogpt* function for selection. Because such recombinant genomes can be propagated in bacterial cells, selected regions of the DNA can be modified *in vitro*, characterized after recloning in bacteria, and then tested in the appropriate cells by cotransformation for *Ecogpt*.

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